Description

THYMIDINE KINASE MUTANTS AND FUSION PROTEINS HAVING THYMIDINE KINASE AND GUANYLATE KINASE ACTIVITIES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of United States Provisional Application No. 60/061,812, filed October 14, 1997, which application is incorporated by reference in its entirety.

TECHNICAL FIELD

The present invention relates generally to mutant enzymes of the *Herpesviridae* and, more specifically, to compositions and methods which utilize thymidine kinase mutants. The present invention also relates to fusion proteins having both guanylate kinase and thymidine kinase activities.

BACKGROUND OF THE INVENTION

Although many bacterial diseases are, in general, easily treated with antibiotics, very few effective treatments exist for many viral, parasitic, cancerous, and genetic diseases. Cancer, for example, may be treated by surgical resection of a solid tumor. Nevertheless, a majority of patients with solid tumors also possess micrometastases beyond the primary tumor site. If treated with surgery alone, approximately 70% of these patients will experience recurrence of the cancer. Thus, cancer accounts for one-fifth of the total mortality in the United States, and is the second leading cause of death.

In addition to surgery, many cancers are now also treated with a combination of therapies involving cytotoxic chemotherapeutic drugs (*e.g.*, vincristine, vinblastine, cisplatin, methotrexate, 5-FU, etc.) and/or radiation therapy. One difficulty with this approach, however, is that radiotherapeutic and chemotherapeutic agents are toxic to normal tissues, and often create life-threatening side effects. In addition, these approaches often have extremely high failure/remission rates (up to 90% depending upon the type of cancer).

Numerous other methods have been attempted in order to bolster or augment an individual's own immune system in order to eliminate cancer cells. For example, some scientists have utilized bacterial or viral components as adjuvants, in order to stimulate the immune system to destroy tumor cells. Such agents have generally been useful as adjuvants

and as nonspecific stimulants in animal tumor models, but have not yet proved to be generally effective in humans.

Lymphokines have also been utilized in the treatment of cancer (as well as viral and parasitic diseases), in order to stimulate or affect specific immune cells in the generation of an immune response. One group, for example, utilized the lymphokine Interleukin-2 in order to stimulate peripheral blood cells in order to expand and produce large quantities of cells which are cytotoxic to tumor cells (Rosenberg et al., *N. Engl. J. Med.* 313:1485-1492, 1985).

Others have suggested the use of antibody-mediated treatment using specific monoclonal antibodies or "magic bullets" in order to specifically target and kill tumor cells (Dillman, "Antibody Therapy," Principles of Cancer Biotherapy, Oldham (ed.), Raven Press, Ltd., New York, 1987). One difficulty, however, is that most monoclonal antibodies are of murine origin, and thus hypersensitivity against the murine antibody may limit its efficacy, particularly after repeated therapies. Common side effects include fever, sweats and chills, skin rashes, arthritis, and nerve palsies.

One approach which has recently garnered significant interest is the use of gene therapy, which has been utilized to treat not only genetic diseases, but viral and cancerous diseases as well (see PCT Publication Nos. WO 91/02805, EPO 415,731, and WO 90/07936). Briefly, specifically designed vectors which have been derived from viruses are used to deliver particular genetic information into cells. Such genetic information may itself be useful to block expression of damaging proteins or antigens (*e.g.*, antisense therapy), may encode proteins which are toxic and kill selected cells, may encode therapeutic proteins which bolster a cell's immune response, or encode proteins which replace inactive or nonexistent proteins.

One protein which has recently been suggested for use in such therapies is the type 1 Herpes Simplex Virus thymidine kinase (HSVTK-1). Briefly, thymidine kinase is a salvage pathway enzyme which phosphorylates natural nucleoside substrates as well as nucleoside analogues (see Balasubramaniam et al., *J. of Gen. Vir. 71:*2979-2987, 1990). This protein may be utilized therapeutically by introducing a retroviral vector which expresses the protein into the cell, followed by administration of a nucleoside analogue such as acyclovir or ganciclovir. HSVTK-1 then phosphorylates the nucleoside analogue, creating a toxic product capable of killing the host cell. Thus, use of retroviral vectors which express HSVTK has been suggested for not only the treatment of cancers, but for other diseases as well.

The present invention provides novel thymidine kinase mutants and TK fusion proteins with enhanced biological activities which are suitable for a variety of applications, such as gene therapy, and further provides other, related advantages.

SUMMARY OF THE INVENTION

Briefly stated, the present invention provides compositions and methods which utilize *Herpesviridae* thymidine kinase mutants. Within one aspect of the present invention, isolated nucleic acid molecules which encode *Herpesviridae* thymidine kinase enzymes comprising one or more mutations are provided, wherein at least one of the mutations encoding an amino acid substitution is positioned within the Q substrate binding domain, wherein the mutation increases a biological activity of the thymidine kinase, as compared to unmutated thymidine kinase. Within another aspect of the present invention, isolated nucleic acid molecules are provided encoding a *Herpesviridae* thymidine kinase enzyme comprising at least three mutations, at least two of the mutations being amino acid substitutions located toward the N-terminus from a DRH nucleoside binding site (*e.g.*, 1, 2 or 3 amino acids toward the N-terminus), and at least one mutation located toward the C-terminus from a DRH nucleoside binding site (*e.g.*, *4* or 5 amino acids toward the C-terminus) which increases a biological activity of the thymidine kinase, as compared to unmutated thymidine kinase. Representative examples of suitable *Herpesviridae* thymidine kinase enzymes include Herpes Simplex Virus Type 1 thymidine kinase, Herpes Simplex Virus Type 2 thymidine kinase, Varicella Zoster Virus thymidine kinase, and marmoset herpesvirus, feline herpesvirus type 1, pseudorabies virus, equine herpesvirus type 1, bovine herpesvirus type 1, turkey herpesvirus, Marek's disease virus, herpesvirus saimiri and Epstein-Barr virus thymidine kinases. Within other embodiments, the thymidine kinase may be a primate herpesvirus thymidine kinase, or a non-primate herpesvirus thymidine kinase, such as an avian herpesvirus thymidine kinase.

A wide variety of mutations are contemplated within the context of the present invention. For example, within one embodiment mutations, such as amino acid substitutions, may occur within a region that includes the Q substrate binding domain and an additional 11 amino acids from this domain, toward the N-terminus.

In other embodiments, at least one mutation occurs within this "expanded" Q substrate binding domain or within the Q substrate binding domain, and at least one mutation is present outside these two regions. For example, one or more additional mutations may be located within a DRH nucleoside binding site which increases a biological activity of said thymidine kinase, as compared to unmutated thymidine kinase. For example, glutamic acid

may be substituted for aspartic acid in the DRH nucleoside binding site, or a histidine residue may be substituted for arginine in the DRH nucleoside binding site.

Within yet another aspect, isolated nucleic acid molecules are provided encoding *a Herpesviridae* thymidine kinase enzyme comprising at least one mutation, such as an amino acid substitution, within a Q substrate binding domain (or within an expanded Q substrate binding domain) and at least one additional mutation being an amino acid substitution located toward the C-terminus from a DRH nucleoside binding site (*e.g.*, 4, 5 or 6 amino acids toward the C-terminus) which increases a biological activity of the thymidine kinase, as compared to unmutated thymidine kinase.

Alternatively, additional mutations may encode one or more amino acid substitutions located from 1 to 7 amino acids toward the N-terminus from the DRH nucleoside binding site. For example, the amino acid which is one position toward the N-terminus from the DRH nucleoside binding site is substituted with an amino acid selected from the group consisting of valine, leucine, cysteine and isoleucine. Within another embodiment, the amino acid alanine is substituted for the amino acid which is present seven amino acids toward the N-terminus from the DRH nucleoside binding site. Within other embodiments, the thymidine kinase enzyme is truncated, and yet retains biological activity.

Within further embodiments of the invention, isolated nucleic acid molecules are provided which encode a thymidine kinase enzyme capable of phosphorylating a nucleoside analogue (*e.g.*, acyclovir or ganciclovir) at least one-fold over the phosphorylation of the nucleoside analogue by a wild-type thymidine kinase enzyme. Within other embodiments, the thymidine kinase enzyme phosphorylates a nucleoside analogue at least x-fold over the phosphorylation of a nucleoside analogue by a wild-type thymidine kinase enzyme, wherein x is selected from the group consisting of 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5. Within yet another embodiment, the thymidine kinase enzyme is capable of phosphorylating a nucleoside analogue, wherein

$$z < \left[\frac{(TKmNAp)/TKmTp)}{(TKwtNAp)/(TKwtTp)} \right]$$

and wherein $TK_m\ NA_p$ is the rate of phosphorylation of a nucleoside analogue by a thymidine kinase mutant, $TK_m\ T_p$ is the rate of phosphorylation of thymidine by a thymidine kinase mutant, $TK_{wt}\ NA_p$ is the rate of phosphorylation of a nucleoside analogue by an unmutated thymidine kinase enzyme, $TK_{wt}\ T_p$ is the rate of phosphorylation of a thymidine kinase enzyme by an unmutated thymidine kinase enzyme, and z is selected from the group

consisting of 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5. Representative examples of suitable nucleoside analogues include ganciclovir, acyclovir, famciclovir, buciclovir, penciclovir, valciclovir, trifluorothymidine, 1-[2-deoxy, 2-fluoro, beta-D-arabino furanosyl]-5-iodouracil, ara-A, araT 1-beta-D-arabinofuranoxyl thymine, 5-ethyl-2'-deoxyuridine, 5-iodo-5'-amino-2, 5'-dideoxyuridine, idoxuridine, AZT, AIU, dideoxycytidine and AraC.

Particularly preferred mutant thymidine kinases for the increased phosphorylation of nucleoside analogues include those wherein the enzyme is a type 1 Herpes Simplex Virus thymidine kinase.

Within other aspects of the present invention, mutant thymidine kinase enzymes which are encoded by the above-described nucleic acid molecules are provided, as well as vectors which are capable of expressing such molecules. Within one aspect, expression vectors are provided comprising a promoter operably linked to a nucleic acid molecule of the present invention. Within a preferred aspect, the vector is a viral vector capable of directing the expression of a nucleic acid molecule as described above. Representative examples of such viral vectors include herpes simplex viral vectors, adenoviral vectors, adenovirus-associated viral vectors, pox vectors, parvoviral vectors, baculovirus vectors and retroviral vectors. Within another aspect, viral vectors are provided which are capable of directing the expression of a nucleic acid molecule which encodes a thymidine kinase enzyme comprising one or more mutations, at least one of the mutations encoding an amino acid substitution which increases a biological activity of thymidine kinase, as compared to unmutated thymidine kinase.

A wide variety of promoters may be utilized in the present invention, including, for example, promoters such as the MoMLV LTR, RSV LTR, Friend MuLv LTR, Adenoviral promoter, Neomycin phosphotransferase promoter/enhancer, late parvovirus promoter, Herpes TK promoter, SV40 promoter, Metallothionen IIa gene enhancer/promoter, Cytomegalovirus Immediate Early Promoter, Cytomegalovirus Immediate Late Promoter, as well as tissue-specific promoters such as the tyrosinase related promoters (TRP-1 and TRP-2), DF3 enhancer , SLPI promoter (secretory leucoprotease inhibitor -- expressed in many types of carcinomas), TRS (tissue specific regulatory sequences), tyrosine hydroxylase promoter, adipocyte P2 promoter, PEPCK promoter, CEA promoter, α fetoprotein promoter, whey acidic promoter, and casein promoter. Within related aspects, the above-described vectors may be provided as pharmaceutical compositions, along with a pharmaceutically acceptable carrier or diluent.

The present invention further provides nucleic acid molecules encoding fusion proteins that comprise a thymidine kinase moiety and a guanylate kinase moiety. Such fusion

proteins possess biological activities of both thymidine kinase and guanylate kinase. The thymidine kinase moiety may derived from a wild-type thymidine kinase or from one of the thymidine kinase mutants described herein.

Within further aspects, sequences which encode thymidine kinase mutants, thymidine kinase fusion proteins, or fusion proteins having guanylate kinase and thymidine kinase activities described herein may be included within a given vector which is utilized for the purposes of gene therapy. Cells which contain these vectors may subsequently be killed by administration of a nucleoside analogue, in order to prevent formation of replication competent virus or aberrant integration of the vector into the host cell. Such compositions or methods are referred to as "suicide vectors" or a "failsafe" approach to gene therapy.

Within other aspects of the present invention, host cells are provided which carry one of the above-described vectors. Representative examples of such cells include human cells, dog cells, monkey cells, rat cells, and mouse cells.

Within other aspects of the present invention, methods are provided for inhibiting a pathogenic agent in a warm-blooded animal, comprising the step of administering to a warm-blooded animal a vector as described above, such that the pathogenic agent is inhibited. Within various embodiments, the vector may be administered *in vivo,* or to cells *ex vivo,* which are then transplanted (or re-transplanted) in the animal. Within other embodiments, the pathogenic agent may be viruses, bacteria, parasites, tumor cells, or autoreactive immune cells.

Within other aspects of the present invention, methods are provided for noninvasive monitoring of the activity of herpes virus thymidine kinase activity, such as for the monitoring of the progress of gene therapy using herpes virus thymidine kinase. According to such methods, a subject, who has received a vector comprising a herpes virus thymidine kinase, is scanned (*e.g.*, using a clinical gamma camera or by singlephoton emission tomography) for radiolabeled anti-viral drugs that are substrates for the thymidine kinase.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions *(e.g.*, plasmids, *etc.),* and are therefore incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic outline which depicts a strategy for construction of a random nucleotide-containing library, and selection of TK mutants.

Figure 2 is a photograph which shows selection of TK and AZT mutants.

Figure 3 depicts the nucleic acid and amino acid sequences of: Wildtype, TKF 105 (SEQ ID NOs. 55 and 56), TK1208, and ATKF2 TK for codons 165 to 175.

Figure 4 is a series of graphs which depict the thermostability of wildtype TK and TK mutants.

Figure 5 is a graph which depicts heat-inactivation profiles for *in vitro* translated wild-type and TKF2 thymidine kinase.

Figure 6 is an autoradiograph of SDS/PAGE-fractionated *in vitro* translated products (wild-type and TKF2).

Figure 7 is an autoradiograph of ^{35}S-radiolabeled cell-free translation products subjected to SDS-PAGE and TCA-precipitable counts.

Figures 8A and 8B are two graphs which illustrate a time course analysis of high activity(A) and low activity (B) mutants produced in a rabbit reticulocyte lysate cell-free translation system.

Figures 9A and 9B are two graphs which show the thermal stability of high activity (A) and low activity (B) TK mutants.

Figure 10 is a bar graph which depicts a phosphorylation of nucleosides and nucleoside analogs by mutant and wild-type thymidine kinases.

Figure 11 is a bar graph which indicates TK activity of wild-type, TKF36, and dummy (pMDC) plasmids.

Figure 12 is a graph which indicates the thymidine uptake activity of cells containing TKF36, TKF52, wild-type plasmid, TKF99 (SEQ ID NOs. 20 and 21), or dummy plasmids (pMDC) over time.

Figure 13 is a schematic illustration of one representative example of gene therapy utilizing an HSVTK mutant.

Figure 14 is an illustration which depicts the nucleotides which were randomized in the LIF-ALL library, as well as the results of selection.

Figure 15 is a table which shows amino acid substitutions of selected and unselected clones.

Figure 16 is a table which shows the number of mutants selected from the LIF-ALL library which were sensitive to GCV or ACV.

Figure 17 is a table which shows nucleotide changes in selected TK mutants.

Figure 18 is a table which shows the amino acid sequence at positions 159-161 and 168-170, and phosphorylation level of several mutant TKs.

Figure 19 is a graph which shows the survival of cells grown on GCV and transfected with various TK mutants.

Figure 20 is a graph which shows the survival of cells grown on ACV and transfected with various TK mutants.

Figure 21 shows semi-randomized oligonucleotides used to generate a second generation of TK mutants having amino acid substitutions in residues 159-161 and 168-169.

Figure 22 illustrates the use of particular oligonucleotides to construct TK mutants having amino acid substitutions in residues 112-132.

Figure 23 shows nucleotides in the open reading frame of HSVTK-1 (SEQUENCE ID No. 1).

Figure 24 illustrates a nucleotide sequence and deduced amino acid sequence representative of a human guanylate kinase.

Figure 25 illustrates a nucleotide sequence and deduced amino acid sequence of a representative murine guanylate kinase.

Figure 26 is a graph which shows the sensitivity of TK clones to GCV.

Figure 27 is a graph which shows the sensitivity of TK clones to ACV.

Figure 28 is a graph which shows the sensitivity of guanylate kinase transfectant pools to GCV in TK expressing clones.

Figure 29 is a graph which shows the sensitivity of guanylate kinase transfectant pools to ACV in TK expressing clones.

Figure 30 is an illustration of gmk/TK fusion protein constructs.

Figure 31 is a graph which shows a ganciclovir dose response curve, comparing wild-type TK with a gmk/TK fusion protein.

Figure 32 is a graph which shows tumor growth after transfection by various vectors, and subsequent exposure to ACV.

Figure 33 is a graph which shows tumor growth after transfection by various vectors, and subsequent exposure to GCV.

Figure 34 is a bar graph which shows the percentage change of tumor weight for various treatments.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

"Vector" refers to an assembly which is capable of directing the expression of the mutant *tk* gene, as well as any additional sequence(s) or gene(s) of interest. The vector must include transcriptional promoter/enhancer elements, as well as another sequence which, when transcribed, is operably linked to the *tk* gene and/or other gene of interest. The vector may be composed of either deoxyribonucleic acids ("DNA"), ribonucleic acids ("RNA"), or a combination of the two (*e.g.*, a DNA-RNA chimeric). Optionally, the vector may include a polyadenylation sequence, one or more restriction sites, as well as one or more selectable markers such as neomycin phosphotransferase or hygromycin phosphotransferase. Additionally, depending on the host cell chosen and the vector employed, other genetic elements such as an origin of replication, additional nucleic acid restriction sites, enhancers, sequences conferring inducibility of transcription, and selectable markers, may also be incorporated into the vectors described herein.

"Tissue-specific promoter" refers to transcriptional promoter/enhancer elements which control gene expression in a limited number of tissues, or in a single tissue. Representative examples of tissue-specific promoters include the tyrosine hydroxylase promoter, adipocyte P2 promoter, PEPCK promoter, α fetoprotein promoter, whey acidic promoter, and casein promoter.

"Biological activity of thimidine kinase" refers to the ability of the thymidine kinase enzyme to phosphorylate nucleosides (*e.g.*, dT) and nucleoside analogues such as ganciclovir (9-{[2-hydroxy-l-(hydroxymethyl)ethoxyl methyl} guanosine), famciclovir, buciclovir, penciclovir, valciclovir, acyclovir (9-[2-hydroxy ethoxy)methyl] guanosine), trifluorothymidine, 1-[2-deoxy, 2-fluoro, beta-D-arabino furanosyl]-5-iodouracil, ara-A (adenosine arabinoside, vivarabine), 1-beta-D-arabinofuranoxyl thymine, 5-ethyl-2'-deoxyuridine, 5-iodo-5'-ammo-2,5'-dideoxyuridine, idoxuridine (5-iodo-2'-deoxyuridine), AZT (3' azido-3' thymidine), ddC (dideoxycytidine), AIU (5-iodo-5' amino 2', 5'-dideoxyuridine) and AraC (cytidine arabinoside). As utilized herein, a thymidine kinase mutant is considered to have "increased biological activity" if the level or rate of activity increases at least "y" fold over unmutated thymidine kinase, wherein y is selected from the group consisting of 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5. Within preferred embodiments, thymidine kinase mutants are considered to have increased biological activity when

$$z < \left[\frac{(TKmNAp)/TKmTp)}{(TKwtNAp)/(TKwtTp)} \right]$$

wherein $TK_m\ NA_p$ is the rate of phosphorylation of a nucleoside analogue by a thymidine kinase mutant, $TK_m\ T_p$ is the rate of phosphorylation of thymidine by a thymidine kinase mutant, $TK_{wt}\ NA_p$ is the rate of phosphorylation of a nucleoside analogue by an unmutated thymidine kinase enzyme, $TK_{wt}\ T_p$ is the rate of phosphorylation of a thymidine kinase enzyme by an unmutated thymidine kinase enzyme, and z is selected from the group consisting of 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5.

"Biological activity of guanylate kinase" refers to the ability of the guanylate kinase enzyme to catalyze the reversible transfer of the terminal phosphoryl group of ATP to an acceptor molecule such as GMP or dGMP. Guanylate kinase (gmk) can also phosphorylate nucleosides and nucleoside analogs that have been phosphorylated by thymidine kinase. Examples of thymidine kinase substrates are described above.

In addition to the ability of thymidine kinase and guanylate kinase to phosphorylate nucleosides and nucleoside analogues, the phrase "biological activity" should also be understood to refer to other biological properties of these enzymes, such as protein stability (*e.g.*, as measured by resistance to proteolytic enzyme degradation by enzymes such as trypsin), and thermostability (*e.g.*, maintenance of nucleoside analogue phosphorylation upon increases in temperature).

"Pathogenic agent" refers to either a foreign organism which is responsible for a disease state, or an "altered" cell which is responsible for a disease state. Representative examples of pathogenic agents include foreign organisms such as viruses, bacteria and parasites, as well as altered cells such as tumor cells and autoreactive immune cells. As utilized herein, a pathogenic agent is considered to be "inhibited" if either the growth or spread of the pathogenic agent is slowed, or if the pathogenic agent itself is destroyed.

As noted above, the present invention provides compositions and methods which utilize *Herpesviridae* thymidine kinase mutants. Briefly, thymidine kinase mutants of the present invention may be prepared from a wide variety of *Herpesviridae* thymidine kinases, including for example both primate herpesviruses, and nonprimate herpesviruses such as avian herpesviruses. Representative examples of suitable herpesviruses include Herpes Simplex Virus Type 1 (McKnight et al., *Nuc. Acids Res 8*:5949-5964, 1980), Herpes Simplex Virus Type 2 (Swain and Galloway, *J. Virol. 46*:1045-1050, 1983), Varicella Zoster Virus (Davison and Scott, *J. Gen. Virol. 67*:1759-1816, 1986), marmoset herpesvirus (Otsuka and

Kit, *Virology 135*:316-330, 1984), feline herpesvirus type 1 (Nunberg et al., *J. Viral. 63*:3240-3249, 1989), pseudorabies virus (Kit and Kit, U.S. Patent No. 4,514,497, 1985), equine herpesvirus type 1 (Robertson and Whalley, *Nuc. Acids Res. 16*:11303-11317, 1988), bovine herpesvirus type 1 (Mittal and Field, *J. Virol 70*:2901-2918, 1989), turkey herpesvirus (Martin et al., *J. Virol. 63*:2847-2852, 1989), Marek's disease virus (Scott et al., *J. Gen. Virol. 70*:3055-3065, 1989), herpesvirus saimiri (Honess et al., *J. Gen. Virol. 70*:3003-3013, 1989) and Epstein-Barr virus (Baer et al., *Nature (London) 310*:207-311, 1984).

Such herpesviruses may be readily obtained from commercial sources such as the American Type Culture Collection ("ATCC", Rockville, Maryland). Deposits of certain of the above-identified herpesviruses may be readily obtained from the ATCC, for example: ATCC No. VR-539 (Herpes simplex type 1); ATCC Nos. VR-734 and VR-540 (Herpes Simplex type 2); ATCC No. VR-586 (Varicella Zoster Virus); ATCC No. VR-783 (Infectious laryngothracheitis); ATCC Nos. VR-624 VR-987, VR-2103, VR-2001, VR-2002, VR-2175, VR-585 (Marek's disease virus); ATCC Nos. VR-584B and VR-584B (turkey herpesvirus); ATCC Nos. VR-631 and VR-842 (bovine herpesvirus type 1); and ATCC Nos. VR-2003, VR-2229 and VR-700 (equine herpesvirus type 1). Herpesviruses may also be readily isolated and identified from naturally occurring sources (*e.g.*, from an infected animal).

Any of the above-cited herpesviruses (as well as other members of the *Herpesviridae*) may be readily utilized in order to prepare thymidine kinase mutants of the present invention. Briefly, one primary region which is believed to be responsible for nucleoside binding is found in the area surrounding Sites 3 and 4 (*see* Balasubramaniam et al., *J. Gen. Vir. 71*:2979-2987, 1990). These sites are characterized by highly conserved regions, and consist of the motif -DRH- (for Site 3), and -C(Y/F)P- (for Site 4). Although the numbering of nucleic acids may change substantially from one herpesvirus to another, as utilized herein, reference will be made to positions relative to the DRH nucleoside binding site. For example, for Herpes Simplex Virus type 1 (McKnight et al., *Nucl. Acids Res. 8*:5949-5964, 1980), this site may be found at amino acids 162, 163 and 164. DRH nucleoside binding sites for other representative herpesviruses include: 163, 164 and 165 for Herpes Simplex Virus type 2; 129, 130 and 131 for Varicella Zoster Virus; 130, 131 and 132 for Marmoset herpesvirus; and 148, 149 and 150 for Epstein-Barr virus.

For herpesviruses which have not been previously sequenced, the DRH nucleoside binding site may be readily identified by sequencing the nucleic acid sequence encoding the enzyme, or by amino acid sequencing the enzyme itself, followed by alignment of the sequence to other known herpesvirus sequences (*see* Balasubramanian, *ibid.*). To the extent that more than one -DRH- motif is identified, the proper motif may be readily

identified by, for example, crystal structure analysis (Sanderson et al., *J. Mol. Biol. 202*:917-919, 1988; Montfort et al., *Biochem 29*(30):6964-6977, 1990; Hardy et al., *Science 235*:448-455, 1987), or crosslinking studies (Knoll et al., *Bioch. Biophys. Acta 1121*:252-260, 1992).

The thymidine kinase gene from the selected herpesvirus may then be readily isolated and mutated as described below, in order to construct nucleic acid molecules encoding a thymidine kinase enzyme comprising one or more mutations which increases a biological activity of the thymidine kinase, as compared to unmutated thymidine kinase. As utilized herein, it should be understood that "unmutated thymidine kinase" refers to native or wild-type thymidine kinase such as that described by McKnight et al. (*Nucl. Acids Res. 8*:5949-5964, 1980). The biological activity of such kinases may be readily determined utilizing any of the assays which are described herein, including for example, determination of the rate of nucleoside analogue uptake, determination of the rate of nucleoside or nucleoside analogue phosphorylation (see Examples 2-4). In addition, thymidine kinase mutants may be readily selected which are characterized by other biological properties, such as thermostability (see Examples 2-4), and protein stability.

A wide variety of thymidine kinase mutations are contemplated within the scope of the present invention. For example, within one embodiment of the invention, isolated nucleic acid molecules are provided which encode a *Herpesviridae* thymidine kinase enzyme comprising one or more mutations, at least one of the mutations encoding an amino acid substitution located toward the N-terminus from the DRH nucleoside binding site. Briefly, any amino acid position toward the N-terminus of the DRH nucleoside binding site may be substituted for another amino acid given the disclosure provided herein. Representative amino acids which may be substituted (and their one letter symbols) include alanine (A), arginine (R), asparagine (N), aspartic acid (D), cysteine (C), glutamine (Q), glutamic acid (E), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).

For example, within one embodiment of the invention, isolated nucleic acid molecules are provided which encode a *Herpesviridae* thymidine kinase enzyme comprising at least three mutations, at least two of the mutations being amino acid substitutions located toward the N-terminus from a DRH nucleoside binding site (*e.g.*, 1, 2 or 3 amino acids toward the N-terminus), and at least one mutation located toward the C-terminus from a DRH nucleoside binding site (*e.g.*, 4 or 5 amino acids toward the C-terminus) which increases a biological activity of the thymidine kinase, as compared to unmutated thymidine kinase. Briefly, an amino acid in any of these positions may be substituted for another amino acid given the disclosure provided herein. Representative amino acids which may be substituted

(and their one letter symbols) include alanine (A), arginine (R), asparagine (N), aspartic acid (D), cysteine (C), glutamine (Q), glutamic acid (E), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V). With reference to TK mutants having at least two mutations toward the N-terminus and at least one mutation toward the C-terminus from a DRH site, preferred amino acids that may be substituted for amino acids of a wild-type sequence include alanine (A), asparagine (N), isoleucine (I), leucine (L), methionine (M), phenylalanine (F), tyrosine (Y), and valine (V).

Within another embodiment of the invention, nucleic acid molecules are provided which encode thymidine kinase mutants either with one or more amino acid substitutions within the Q substrate binding domain, or with one or more amino acid substitutions within an expanded region that includes the Q substrate binding domain and an additional 11 amino acid residues located toward the N-terminus ("the expanded Q substrate binding domain"). Representative amino acids which may be substituted (and their one letter symbols) include alanine (A), arginine (R), asparagine (N), aspartic acid (D), glutamine (Q), glutamic acid (E), glycine (G), histidine (H), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), and tyrosine (Y).

Within another embodiment, nucleic acid molecules are provided which encode thymidine kinase mutants having with one or more amino acid substitutions within the Q substrate binding domain or within the expanded Q substrate binding domain, and at least one additional amino acid substitution located from two to six positions toward the N-terminus from the DRH nucleoside binding site. Representative amino acids which may be substituted include alanine (A), arginine (R), asparagine (N), aspartic acid (D), cysteine (C), glutamine (Q), glutamic acid (E), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).

Within other embodiments, nucleic acid molecules are provided which encode thymidine kinase mutants having with one or more amino acid substitutions within the Q substrate binding domain or within the expanded Q substrate binding domain, and at least one additional amino acid substitution located seven positions toward the N-terminus from the DRH nucleoside binding site. Representative amino acids which may be substituted include arginine (R), asparagine (N), aspartic acid (D), cysteine (C), glutamine (Q), glutamic acid (E), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).

Within other aspects of the invention, nucleic acid molecules are provided which encode thymidine kinase mutants having with one or more amino acid substitutions within the Q substrate binding domain or within the expanded Q substrate binding domain, and at least one additional mutation, as described by Dedieu et al., international publication No. WO 95/14102, which is hereby incorporated by reference.

Within another aspect of the present invention, nucleic acid molecules are provided which encode thymidine kinase mutants having with one or more amino acid substitutions within the Q substrate binding domain or within the expanded Q substrate binding domain, and at least one additional amino acid substitution within the DRH nucleoside binding site. Within one embodiment of the invention, the asparatic acid in the DRH nucleoside binding site is substituted with other amino acids, including for example, alanine (A), arginine (R), asparagine (N), cysteine (C), glutamine (Q), glutamic acid (E), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V). Within another embodiment of the invention, the arginine in the DRH nucleoside binding site is substituted with other amino acids, including for example, alanine (A), asparagine (N), aspartic acid (D), cysteine (C), glutamine (Q), glutamic acid (E), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).

Within other aspects of the present invention, nucleic acid molecules are provided which encode thymidine kinase enzymes comprising two or more mutations which increase a biological activity of the thymidine kinase enzyme, wherein the mutants have one or more amino acid substitutions within the Q substrate binding domain or within the expanded Q substrate binding domain, and one or more amino acid substitutions located 1, 2 or 3 amino acids toward the N-terminus from the DRH nucleoside binding site, and/or one or more substitutions located 4, 5 or 6 amino acids toward the C-terminus from the DRH nucleoside binding site, or located 1, 2 or 3 amino acids toward the N-terminus from the CYP nucleoside binding site (*see* Figure 14).

Within yet another embodiment of the invention, thymidine kinase mutants are characterized by having one or more amino acid substitutions within the Q substrate binding domain or within the expanded Q substrate binding domain, and by having the histidine in the DRH nucleoside binding site substituted with any other amino acid, including for example, alanine (A), arginine (R), asparagine (N), aspartic acid (D), cysteine (C), glutamine (Q), glutamic acid (E), glycine (G), isoleucine (1), leucine (L), lysine (K), methionine (M),

phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).

Within other aspects of the present invention, nucleic acid molecules are provided which encode thymidine kinase enzymes comprising two or more mutations which increase a biological activity of the thymidine kinase enzyme, wherein one or more amino acid substitutions are located within the Q substrate binding domain or within the expanded Q substrate binding domain, and wherein at least one mutation encodes an amino acid substitution located from 1 to 11 positions toward the C-terminus from the DRH nucleoside binding site. These amino acids may be substituted with other amino acids, including for example, alanine (A), arginine (R), asparagine (N), aspartic acid (D), cysteine (C), glutamine (Q), glutamic acid (E), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).

Within another aspect of the present invention, nucleic acid molecules are provided which encode thymidine kinase enzymes comprising one or more mutations which increase a biological activity of the thymidine kinase enzyme, wherein one or more amino acid substitutions are located within the Q substrate binding domain or within the expanded Q substrate binding domain, and wherein at least one mutation encodes an amino acid substitution located from 12 to "v" positions toward the C-terminus from the DRH nucleoside binding site, wherein "v" is any integer greater than 13 (and generally less than 202). These amino acids may be readily substituted with other amino acids, including for example, alanine (A), arginine (R), asparagine (N), aspartic acid 04 cysteine (C), glutamine (Q), glutamic acid (E), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).

Within various aspects, nucleic acid molecules of the present invention may encode several amino acid mutations. For example, within one preferred embodiment, thymidine kinase mutants are provided which encode mutations with 1, 2, 3, 4, 5 or more amino acid substitutions, as well as in-frame deletions. Example of such mutants include P155A/F161V (SEQ ID NO. 141), P155A/F161C (SEQ ID NO. 158), P155A/D162E (SEQ ID NO. 159), I160L/F161L/A168V/L169M (SEQ ID NO. 160) and F161L/A168V/L169Y/L170C (SEQ ID NO. 161).

As described herein, mutagenesis of nucleotides encoding the residues surrounding Sites 3 and 4 of HSV-1 TK has lead to improvements in the kinetic parameters (Km) towards nucleoside prodrugs. A new and distinct region has been recently identified to

participate in nucleoside binding that resides within amino acid residues 112-132. The region encoding residues 112-132 of HSV-1 TK was implicated in substrate (or dTMP) binding by photoaffinity labeling using a 32P-azido-dUMP probe (Rechtin et al., *Anal. Biochem.* *237*:135-140, 1996). This initial identification was supported by the observed proximity of these residues to bound substrate (thymidine or ganciclovir), as determined by X-ray crystallography studies (Wild et al., *FEBS Lett.* *368*:289-292, 1995; Brown et al., *Nature Struct. Biol.* *2*:876-881, 1995). Since the glutamine ("Q") residue shows significant conservation in TK enzymes from a wide variety of sources (see, for example, Balasubramaniam et al., *J. Gen. Virol.* *71*:2979-2987, 1990), the region of amino acid residues 112-132 is designated as the "Q substrate binding domain."

Due to its role in substrate binding, this region is an excellent target for mutagenizing and selecting clones with altered substrate specificities. Such mutants would improve the efficacy and specificity of suicide gene therapy in the presence of specific prodrugs. Moreover, these mutant enzymes can be used for cell lineage ablation, restenosis and selection of homologous recombinants.

Accordingly, the present invention includes nucleic acid molecules encoding forms of TK with at least one mutation within the Q substrate binding domain. The present invention also includes nucleic acid molecules encoding truncated TK enzymes having at least one mutation within the Q substrate binding domain. The present invention further includes mutant TK-encoding nucleic acid molecules with at least one modification in a subregion of the Q substrate binding domain, such as within amino acid residues 123-132, or with at least one mutation in an expanded region that includes the Q substrate binding domain and about 11 additional amino acids toward the N-terminus, (*e.g.*, within amino acid residues 101-132). As an illustration, Example 10 describes methods for the mutagenesis of the region encoding amino acids 112-132 of HSV-1 TK. In this example, TK mutants were constructed that contained 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 mutations within amino acid residues 112-132.

Identification of the Q substrate binding domain, which is distinct from the DRH nucleoside binding site, enables the construction of numerous thymidine kinase mutations. Such TK mutants include those having amino acid substitutions in the Q substrate binding domain with any of the following representative amino acids: alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. Functionally, TK mutants having an alteration in the Q substrate binding domain

are characterized by an increased biological activity of thymidine kinase, as compared with unmutated thymidine kinase.

Although Example 10 illustrates mutagenesis of the HSV-1 TK Q substrate binding domain, the present invention also includes a variety of thymidine kinase mutants having alterations in this domain. Identification of a Q substrate binding domain in various TK enzymes can be achieved by aligning a TK amino acid sequence with the HSV-1 TK sequence. For example, Balasubramaniam et al., *J. Gen. Virol. 71*:2979-2987 (1990), provide such an alignment of the following TK enzymes: HSV-1, HSV-2, marmoset herpesvirus, varicella-zoster virus, feline herpesvirus, pseudorabies virus, equine herpesvirus type 1, bovine herpesvirus type 1, turkey herpesvirus, Marek's disease virus, herpesvirus saimiri, and Epstein-Barr virus.

Alternatively, photoaffinity labeling can be used to identify analogous Q substrate binding domains, using the methods described by Rechtin et al., *Anal. Biochem. 237*:135-140 (1996), which is incorporated by reference. In addition, the identification of a Q substrate binding domain can be verified by crystal structure analysis using standard techniques (see, for example, Wild et al., *FEBS Lett. 368*:289292, 1995; Brown et al., *Nature Struct. Biol. 2*:876-881, 1995; De Winter and Herdewijn, *J. Med. Chem. 39*:4727-4737, 1996). In sum, well-known methods can be used to identify analogous Q substrate binding domains in various thymidine kinases. Preferred sources for mutation of the Q substrate binding domain are Herpesviridae thymidine kinases.

The present invention also provides TK mutants that have mutations in the Q substrate binding domain (or, in the expanded Q substrate binding domain) in addition to at least one mutation associated with the DRH nucleoside binding site, as described above. For example, the present invention contemplates TK mutants having at least one amino acid substitution in the Q substrate binding domain (or, in the expanded Q substrate binding domain) and (1) at least two amino acid substitutions located toward the N-terminus from a DRH nucleoside binding site (*e.g.*, one, two or three amino acids toward the N-terminus) and at least one mutation located toward the C-terminus from a DRH nucleoside binding site (*e.g.*, four or five amino acids toward the C-terminus), (2) one or more amino acid substitutions located from one to seven amino acids toward the N-terminus from a DRH nucleoside binding site, (3) amino acid substitutions that are located two to six positions toward N-terminus from the DRH nucleoside binding site, and (4) one or more amino acid substitutions within the DRH nucleoside binding site. Again, such TK mutants are characterized by an increased biological activity of thymidine kinase, as compared with unmutated thymidine kinase.

Any of the above-described thymidine kinase mutants may be readily screened for increased biological activity, given the assays described herein and below in the Examples.

CONSTRUCTION OF THYMIDINE KINASE MUTANTS

Thymidine kinase mutants of the present invention may be constructed using a wide variety of techniques. For example, mutations may be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes a derivative having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific (or segment specific) mutagenesis procedures may be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Deletion or truncation derivatives of thymidine kinase mutants may also be constructed by utilizing convenient restriction endonuclease sites adjacent to the desired deletion. Subsequent to restriction, overhangs may be filled in, and the DNA religated. Exemplary methods of making the alterations set forth above are disclosed by Sambrook et al. (*Molecular cloning: A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory Press, 1989).

Thymidine kinase mutants may also be constructed utilizing techniques of PCR mutagenesis, chemical mutagenesis (Drinkwater and Klinedinst, *PNAS 83*:3402-3406, 1986), by forced nucleotide misincorporation (*e.g.*, Liao and Wise *Gene 88*:107-111, 1990), or by use of randomly mutagenized oligonucleotides (Horwitz et al., *Genome 3*:112-117, 1989). Preferred methods for constructing thymidine kinase mutants are set forth in more detail below in the Examples.

HSVTK VECTORS

Within the context of the present invention, the term "thymidine kinase mutant" should be understood to include not only the specific protein described herein (as well as the nucleic acid sequences which encode these proteins), but derivatives thereof which may include various structural forms of the primary protein which retain biological activity. For example, a thymidine kinase mutant may be in the form of acidic or basic salts, or in neutral form. In addition, individual amino acid residues may be modified by oxidation or reduction. Furthermore, various substitutions, deletions, or additions may be made to the amino acid or nucleic acid sequences, the net effect of which is to retain or further enhance the increased biological activity of the mutant. Due to code degeneracy, for example, there

may be considerable variation in nucleotide sequences encoding the same amino acid sequence.

Other derivatives of the thymidine kinase mutants disclosed herein include conjugates of thymidine kinase mutants along with other proteins or polypeptides. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins which may be added to facilitate purification or identification of thymidine kinase mutants (see U.S. Patent No. 4,851,341, *see also*, Hopp et al., *Bio/Technology 6*:1204, 1988.)

Within one embodiment of the present invention, truncated derivatives of thymidine kinase mutants are provided. For example, site-directed mutagenesis may be readily performed in order to delete the N-terminal 45 amino acids of a thymidine kinase mutant, thereby constructing a truncated form of the mutant which retains its biological activity.

Mutations in nucleotide sequences constructed for expression of derivatives of thymidine kinase mutants should preserve the reading frame phase of the coding sequences. Furthermore, the mutations will preferably not create complementary regions that could hybridize to produce secondary mRNA structures, such as loops or hairpins, which would adversely affect translation of the receptor mRNA. Such derivatives may be readily constructed using a wide variety of techniques, including those discussed above.

As noted above, the present invention provides recombinant vectors which include either synthetic, or cDNA-derived nucleic acid molecules encoding thymidine kinase mutants or derivatives thereof, which are operably linked to suitable transcriptional or translational regulatory elements. Suitable regulatory elements may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, insect, or plant genes. Selection of appropriate regulatory elements is dependent on the host cell chosen, and may be readily accomplished by one of ordinary skill in the art. Examples of regulatory elements include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal.

Nucleic acid molecules which encode any of the thymidine kinase mutants described above may be readily expressed by a wide variety of prokaryotic and eukaryotic host cells, including bacterial, mammalian, yeast or other fungi, viral, insect, or plant cells. Methods for transforming or transfecting such cells to express foreign DNA are well known in the art (see, *e.g.*, Itakura et al., U.S. Patent No. 4,704,362; Hinnen et al., *PNAS USA 75*:1929-1933, 1978; Murray et al., U.S. Patent No. 4,801,542; Upshall et al., U.S. Patent No. 4,935,349; Hagen et al., U.S. Patent No. 4,784,950; Axel et al., U.S. Patent No. 4,399,216; Goeddel et al., U.S. Patent No. 4,766,075; and Sambrook et al. *Molecular Cloning: A*

Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, 1989; for plant cells see Czako and Marton, *Plant Physiol. 104*:1067-1071, 1994; and Paszkowski et al., *Biotech. 24*:387392, 1992).

Bacterial host cells suitable for carrying out the present invention include *E. coli, B. subtilis, Salmonella typhimurium*, and various species within the genus' *Pseudomonas, Streptomyces*, and *Staphylococcus*, as well as many other bacterial species well known to one of ordinary skill in the art. Representative examples of bacterial host cells include DH5α (Stratagene, LaJolla, California).

Bacterial expression vectors preferably comprise a promoter which functions in the host cell, one or more selectable phenotypic markers, and a bacterial origin of replication. Representative promoters include the ß-lactamase (penicillinase) and lactose promoter system (*see* Chang et al., *Nature 275*:615, 1978), the T7 RNA polymerase promoter (Studier et al., *Meth. Enzymol. 185*:60-89, 1990), the lambda promoter (Elvin et al., *Gene 87*:123-126, 1990), the *trp* promoter (Nichols and Yanofsky, *Meth. in Enzymology 101*:155, 1983) and the *tac* promoter (Russell et al., *Gene 20*: 231, 1982). Representative selectable markers include various antibiotic resistance markers such as the kanamycin or ampicillin resistance genes. Many plasmids suitable for transforming host cells are well known in the art, including among others, pBR322 (*see* Bolivar et al., *Gene 2*:95, 1977), the pUC plasmids pUC18, pUC19, pUC118, pUC119 (*see* Messing, *Meth. in Enzymology 101*:20-77, 1983 and Vieira and Messing, *Gene 19*:259-268, 1982), and pNH8A, pNH16a, pNH18a, and Bluescript M13 (Stratagene, La Jolla, Calif.).

Yeast and fungi host cells suitable for carrying out the present invention include, among others *Saccharomyces pombe*, Saccharomyces *cerevisiae*, the genera *Pichia* or *Kluyveromyces* and various species of the genus *Aspergillus*. Suitable expression vectors for yeast and fungi include, among others, YC_p50 (ATCC No. 37419) for yeast, and the amdS cloning vector pV3 (Turnbull, *Bio/Technology 7*:169, 1989). Protocols for the transformation of yeast are also well known to those of ordinary skill in the art. For example, transformation may be readily accomplished either by preparation of spheroplasts of yeast with DNA (*see* Hinnen et al., *PNAS USA 75*:1929, 1978) or by treatment with alkaline salts such as LiCl (*see* Itoh et al., *J. Bacteriology 153*:163, 1983). Transformation of fungi may also be carried out using polyethylene glycol as described by Cullen et al. (*Bio/Technology 5*:369, 1987).

Mammalian cells suitable for carrying out the present invention include, among others: COS (*e.g.*, ATCC No. CRL 1650 or 1651), BHK (*e.g.*, ATCC No. CRL 6281), CHO (ATCC No. CCL 61), HeLa (*e.g.*, ATCC No. CCL 2), 293 (ATCC No. 1573) and NS-1 cells. Suitable expression vectors for directing expression in mammalian cells generally include a

promoter, as well as other transcriptional and translational control sequences. Common promoters include SV40, MMTV, metallothionein-1, adenovirus E1a, Cytomegalovirus Immediate Early Promoter, and the Cytomegalovirus Immediate Late Promoter.

Protocols for the transfection of mammalian cells are well known to those of ordinary skill in the art. Representative methods include calcium phosphate mediated transfection, electroporation, lipofection, retroviral, adenoviral and protoplast fusion-mediated transfection (*see* Sambrook et al., *supra*).

Thymidine kinase mutants may be prepared by culturing the host/vector systems described above, in order to express the recombinant thymidine kinase mutants. Recombinantly produced thymidine kinase mutants may be further purified as described in more detail below.

As noted above, the present invention also provides a variety of both viral and non-viral vectors which are suitable for directing the expression of the nucleic acid molecules described above. Within one aspect of the invention, viral vectors are provided which comprise a promoter that directs the expression of an isolated nucleic acid molecule which encodes a thymidine kinase mutant as described above. A wide variety of promoters may be utilized within the context of the present invention, including for example, promoters such as MoMLV LTR, RSV LTR, Friend MuLV LTR, Adenoviral promoter (Ohno et al., *Science 265*: 781-784, 1994), Neomycin phosphotransferase promoter/enhancer, late parvovirus promoter (Koering et al., *Hum. Gene Therap. 5*:457-463, 1994), Herpes TK promoter, SV40 promoter, Metallothionein IIa gene enhancer/promoter, Cytomegalovirus Immediate Early Promoter, and the Cytomegalovirus Immediate Late Promoter. Within particularly preferred embodiments of the invention, the promoter is a tissue-specific promoter (see *e.g.*, WO 91/02805; EP 0,415,731; and WO 90/07936). Representative examples of suitable tissue specific promoters include the tyrosinase related promoters (TRP-1 and TRP-2, Vile and Hart, *Canc. Res. 53*:962-967, 1993), DF3 enhancer (for breast cells, *see* Manome et al., *Canc. Res. 54*:5408-5413, 1994), SLPI promoter (secretory leucoprotease inhibitor -- expressed in many types of carcinomas, *see* Garver et al, *Gene Therapy 1*:46-50, 1994), TRS (tissue specific regulatory sequences, *see* Dynan and Tjian, *Nature 316*: 774-778, 1985), albumin and α fetoprotein promoters (specific for normal hepatocytes and transformed hepatocytes, respectively), the carcino-embryonic antigen promoter (for use in transformed cells of the gastrointestinal tract, lung, breast and other tissues), the tyrosine hydroxylase promoter (for melanocytes), choline acetyl transferase or neuron specific enolase promoters for use in neuroblastomas, the regulatory sequence for glial fibroblastomas, the tyrosine hydroxylase promoter, c-*erb* B-2 promoter, PGK promoter, PEPCK promoter, whey acidic promoter

(breast tissue), and casein promoter (breast tissue) and the adipocyte P2 promoter (Ross et al., *Genes & Dev.* 1318-1324, 1993; and Lowell et al., *Nature 366*:740-742, 1993). In addition to the above-noted promoters, other viral-specific promoters (*e.g.*, retroviral promoters (including those noted above, as well as others such as HIV promoters), hepatitis, herpes (*e.g.*, EBV), and bacterial, fungal or parasitic (*e.g.*, malarial) -specific promoters may be utilized in order to target a specific cell or tissue which is infected with a virus, bacteria, fungus or parasite.

Thymidine kinase mutants of the present invention may be expressed from a variety of viral vectors, including for example, adenoviral vectors (*e.g.*, Kass-Eisler et al., *PNAS 90*(24):11498-502, 1993; Kolls et al., *PNAS 91*(1):215-219, 1994; Li et al., *Hum Gene Ther. 4*(4):403-409, 1993; Vincent et al., *Nat. Genet. 5*(2):130-134, 1993; and Zabner et al., *Cell 75*(2):207-216, 1993; WO 94/26914, WO 93/9191), adenovirus-associated viral vectors (Flotte et al., *PNAS 90*(22):10613-10617, 1993), alphaviruses such as Semliki Forest Virus and Sindbis Virus (Hertz and Huang, *J. Vir. 66*(2):857-864, 1992; Raju and Huang, *J. Vir. 65*(5):2501-2510, 1991; Xiong et al., *Science* 243:1188, 1989; U.S. Patent No. 5,091,309; WO 92/10578; WO 95/07994); baculovirus vectors; herpes viral vectors (*e.g.*, U.S. Patent Nos. 4,769,331, 4,859,587, 5,288,641 and 5,328,688; and PCT publication Nos. WO 94/14971 and WO 95/04139), parvovirus vectors (Koering et al., *Hum. Gene Therap. 5*:457-463, 1994), pox virus vectors (Ozaki et al., *Biochem. Biophys. Res. Comm. 193*(2):653-660, 1993; and Panicali and Paoletti, *PNAS 79*:4927-4931, 1982), pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch et al., *PNAS 86*:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci. 569*:86-103, 1989; U.S. Patent Nos. 4,603,112, 4,769,330 and 5,017,487; WO 89/01973); and retroviruses (*e.g.*, Baba et al., *J. Neurosurg 79*:729-735, 1993; Ram et al., *Cancer Res. 53*:83-88, 1993; Takamiya et al., *J. Neurosci. Res 33*:493-503, 1992; Vile and Hart, *Cancer Res. 53*:962-967, 1993; Vile and Hart, *Cancer Res. 53*:3860-3864, 1993; U.S. Patent No. 5,219,740; EP 0,415,731; WO 90/07936; WO 91/0285, WO 94/03622; WO 93/25698; WO 93/25234; WO 93/11230; WO 93/10218). Within various embodiments, either the viral vector itself, or a viral particle which contains the viral vector may be utilized in the methods and compositions described below.

In addition to viral vectors, non-viral vectors systems, or systems which contain portions of a viral vector (*e.g.*, which control transcription, translation, or viral entry into a cell) may be utilized to deliver nucleic acid sequences of the present invention. Representative example of such systems a variety of nucleic acid based transcription systems (*e.g.*, based on T7 or SP6 promoters, *see generally*, Li et al., "Tumor regression in Nude Mice by Direct Injection of a Nonviral Cytoplasmic Gene Expression Vector Containing a

Thymidine Kinase Gene" p. 179, Cold Spring Harbor Meeting in Gene Therapy, Sept. 21-25, 1194; WO 95/07994). Such vector systems may be administered and prepared as described herein (*e.g.*, in liposomes, condensed with polycations, or linked to a ligand).

Vectors of the present invention may contain or express a wide variety of additional nucleic acid molecules in addition to a thymidine kinase nucleic acid molecule as described above. For example, the viral vector may express a lymphokine, antisense sequence, toxin or "replacement" protein (*e.g.*, adenosine deaminase). Representative examples of lymphokines include IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, GM-CSF, G-CSF, M-CSF, alpha-interferon, beta-interferon, gamma interferon, and tumor necrosis factors. Representative examples of antisense sequences include antisense *myc*, antisense p53, antisense *ras*, as well as antisense sequences which block the expression or production of viruses such as HIV, HBV and HCV. Representative examples of toxins include: ricin, abrin, diphtheria toxin, cholera toxin, gelonin, pokeweed antiviral protein, tritin, *Shigella* toxin, and *Pseudomonas* exotoxin A.

Within preferred embodiments of the invention, one or more genes which encode proteins that facilitate or increase the biological activity of thymidine kinase may be included with, and expressed by the vectors described herein. For example, within one embodiment of the invention, nucleic acid molecules which encode DNA polymerase (*e.g.*, a Herpes DNA polymerase) and/or guanylate kinase (Konrad, *J. Biol. Chem. 267*(36):25652-25655, 1992; Miller and Miller, *J. Biol. Chem. 255*(15):7204-7207, 1980) are expressed either from one or several separate promoters (*e.g.*, from multiple internal ribosome binding sites) in addition to a thymidine kinase enzyme (either wild type, or thymidine kinase mutants as described above). Representative examples of such embodiments are set forth in more detail below in Examples 7 and 11. It should be understood that although certain nucleic acid molecules are disclosed which encode DNA polymerase or guanylate kinase, that the present invention is not so limited. Indeed, as discussed above with respect to thymidine kinase mutants, a wide variety of nucleic acid molecules are considered to be included within the scope of the present invention which encode DNA polymerase or guanylate kinase activity (*e.g.*, truncated nucleic acid molecules or nucleic acid molecules which are degenerate with respect to the encoded amino acid sequence).

Thymidine kinase mutants may also be expressed in non-human transgenic animals such as mice, rats, rabbits, sheep, dogs and pigs (*see* Hammer et al. (*Nature 315*:680-683, 1985), Palmiter et al. (*Science 222*:809-814, 1983), Brinster et al. (*Proc. Natl. Acad. Sci. USA 82*:4438-4442, 1985), Palmiter and Brinster (*Cell 41*:343-345, 1985) and U.S. Patent No. 4,736,866). Briefly, an expression unit, including a nucleic acid molecule to be expressed

together with appropriately positioned expression control sequences, is introduced into pronuclei of fertilized eggs, for example, by microinjection. Integration of the injected DNA is detected by blot analysis of DNA from tissue samples. It is preferred that the introduced DNA be incorporated into the germ line of the animal so that it is passed on to the animal's progeny. Tissue-specific expression may be achieved through the use of a tissue-specific promoter, or through the use of an inducible promoter, such as the metallothionein gene promoter (Palmiter et al., 1983, *ibid*), which allows regulated expression of the transgene.

HOST CELLS

The above described nucleic acid molecules which encode thymidine kinase mutants of the present invention (or the vectors which contain and/or express these mutants) may readily be introduced into a wide variety of host cells. Representative examples of such host cells include plant cells, eukaryotic cells, and prokaryotic cells. Within preferred embodiments, the nucleic acid molecules are introduced into cells from a vertebrate or warm-blooded animal, such as a human, macaque, dog, cow, horse, pig, sheep, rat, hamster, mouse or fish cell, or any hybrid thereof.

The nucleic acid molecules (or vectors) may be introduced into host cells by a wide variety of mechanisms, including for example calcium phosphate-mediated transfection (Wigler et al., *Cell 14*:725, 1978), lipofection; gene gun (Corsaro and Pearson, *Somatic Cell Gen. 7*:603, 1981; Graham and Van der Eb, *Virology 52*:456, 1973), electroporation (Neumann et al., *EMBO J. 1*:841-845, 1982), retroviral, adenoviral, protoplast fusion-mediated transfection or DEAE-dextran mediated transfection (Ausubel et al., (eds.), *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc., NY, NY, 1987).

CONSTRUCTION OF GUANYLATE KINASE - THYMIDINE KINASE FUSION PROTEINS

There are several approaches for improving the net efficiency of suicide gene therapy. As described above, one approach is to create novel TK enzymes that efficiently convert systemically delivered prodrugs into cytotoxic compounds. Another strategy is to facilitate the subsequent metabolism of the prodrug to its toxic form by introducing the gene encoding the enzyme responsible for the second step in the nucleotide metabolic pathway of prodrug activation, guanylate kinase, in combination with thymidine kinase. Unlike the cellular thymidine kinase, the HSV TK can perform the initial phosphorylation of produgs, such as GCV and ACV, to their monophosphorylated states. Cellular kinases further phosphorylate the nucleotide to the triphosphate which then inhibits chain elongation by DNA polymerase after insertion into the nascent DNA chain and subsequently leads to cell death.

Guanylate kinase (gmk), the second step in the prodrug activation pathway, appears to be rate limiting *in vivo*. Example 11 illustrates methods for the construction of mammalian expression vectors that produce both gmk and TK enzymes.

In yet another approach, fusion proteins can be constructed that express both gmk and TK enzyme activities, providing the expression of two enzyme functions from a single promoter and a single cistron. In this way, the use of a fusion protein for gene therapy would eliminate the requirement for two promoters, and would eliminate the associated reduction in prodrug activation due to the differences in promoter strength. Moreover, fusion proteins are advantageous for gene therapy vectors which cannot tolerate large pieces of foreign DNA, such as AAV vectors.

Example 12 describes the construction of two gmk-TK fusion proteins. Although the exemplified vectors contain a TK gene fused to the 3'-end of a gmk gene, suitable fusion proteins can be produced with vectors having a gmk gene fused to the 3'-end of a TK gene. Example 12 also illustrates that such fusion proteins need not contain the entire amino acid sequence of a kinase gene. That is, nucleic acid molecules encoding a truncated gmk and/or a truncated TK can be used to express fusion proteins of the present invention. However, such truncated kinases must possess the appropriate biological activity, as defined above. The biological activity of a truncated gmk or a truncated TK can be determined using the enzyme assays described herein.

General methods for producing fusion proteins are well-known to those of skill in the art. See, for example, Ausubel et al. (eds.), *Short Protocols in Molecular Biology*, 3d Edition, pages 16-16 to 16-37 (John Wiley & Sons, Inc. 1995). Example 11 describes methods for obtaining both human and murine gmk clones (also see Brady et al., *J. Biol. Chem. 271*:16734-16740, 1996). Those of skill in the art can obtain nucleic acid molecules encoding gmk from a variety of sources using standard techniques. For example, Konrad, *J. Biol. Chem.* 267:25652-25655 (1992), describes the isolation of gmk sequences from *Saccharomyces cerevisiae*, Gaidarov et al., *FEBS Lett. 335*:81-84 (1993), disclose bovine guanylate kinase sequences, Zschocke et al. *Eur. J. Biochem. 213*:263-269 (1993), provide porcine guanylate kinase sequences, and an *E. coli* guanylate kinase sequence is provided by Gentry et al., *J. Biol. Chem. 268*:14316-14321 (1993). In addition, nucleic acid molecules encoding guanylate kinase enzymes are commercially available. For example, DNA molecules encoding *Mycoplasma genitalium* gmk can be obtained from the American Type Culture Collection (ATCC No. 623592). Suitable TK genes include both known TK genes and the TK mutants of the present invention. Sources for TK genes, suitable expression vectors, and suitable host cells are described above.

PREPARATION OF ANTIBODIES

Antibodies to the thymidine kinase mutants, guanylate kinase protein, or fusion proteins described herein may readily be prepared given the disclosure provided herein. Within the context of the present invention, antibodies are understood to include monoclonal antibodies, polyclonal antibodies, antibody fragments (*e.g.*, Fab, and $F(ab')_2$) as well as portions thereof that may be produced by various recombinant methods. Antibodies are understood to be reactive against a thymidine kinase mutant or fusion protein if it binds with a K_a of greater than or equal to 10^7 M. As will be appreciated by one of ordinary skill in the art, antibodies may be developed which not only bind to a ligand such as a thymidine kinase mutant or fusion protein, but which also block or inhibit the biological activity of the mutant or fusion protein.

Briefly, polyclonal antibodies may be readily generated by one of ordinary skill in the art from a variety of warm-blooded animals such as horses, cows, various fowl, rabbits, mice, or rats. Briefly, a thymidine kinase mutant (or guanylate kinase enzyme, or fusion protein, if such antibodies are desired) is utilized to immunize the animal through intraperitoneal, intramuscular, intraocular, or subcutaneous injections, an adjuvant such as Freund's complete or incomplete adjuvant. Following several booster immunizations, samples of serum are collected and tested for reactivity to the thymidine kinase mutant (or guanylate kinase or fusion protein). Particularly preferred polyclonal antisera will give a signal on one of these assays that is at least three times greater than background. Once the titer of the animal has reached a plateau in terms of its reactivity to the thymidine kinase mutant, guanylate kinase enzyme, or fusion protein, larger quantities of antisera may be readily obtained either by weekly bleedings, or by exsanguinating the animal.

Monoclonal antibodies may also be readily generated using conventional techniques (see U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993 which are incorporated herein by reference; *see also Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Plenum Press, Kennett, McKeam, and Bechtol (eds.), 1980, and *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988, which are also incorporated herein by reference).

Briefly, within one embodiment a subject animal such as a rat or mouse is injected with a thymidine kinase mutant, guanylate kinase enzyme, or fusion protein as described above. The thymidine kinase mutant, guanylate kinase enzyme, or fusion protein may be admixed with an adjuvant such as Freund's complete or incomplete adjuvant in order

to increase the resultant immune response. Between one and three weeks after the initial immunization the animal may be reimmunized with another booster immunization, and tested for reactivity to the thymidine kinase mutant, guanylate kinase enzyme, or fusion protein using assays described above. Once the animal has plateaued in its reactivity to the mutant, it is sacrificed, and organs which contain large numbers of B cells such as the spleen and lymph nodes are harvested.

Cells which are obtained from the immunized animal may be immortalized by transfection with a virus such as the Epstein-Barr virus (EBV) (*see* Glasky and Reading, *Hybridoma 8(4)*:377-389, 1989). Alternatively, within a preferred embodiment, the harvested spleen and/or lymph node cell suspensions are fused with a suitable myeloma cell in order to create a "hybridoma" which secretes monoclonal antibody. Suitable myeloma lines include, for example, NS-1 (ATCC No. TIB 18), and P3X63 - Ag 8.653 (ATCC No. CRL 1580).

Following the fusion, the cells may be placed into culture plates containing a suitable medium, such as RPMI 1640, or DMEM (Dulbecco's Modified Eagles Medium) (JRH Biosciences, Lenexa, Kansas), as well as additional ingredients, such as Fetal Bovine Serum (FBS, *i.e.*, from Hyclone, Logan, Utah, or JRH Biosciences). Additionally, the medium should contain a reagent which selectively allows for the growth of fused spleen and myeloma cells such as HAT (hypoxanthine, aminopterin, and thymidine) (Sigma Chemical Co., St. Louis, Missouri). After about seven days, the resulting fused cells or hybridomas may be screened in order to determine the presence of antibodies which are reactive against a thymidine kinase mutant, guanylate kinase enzyme, or fusion protein. A wide variety of assays may be utilized to determine the presence of antibodies which are reactive against the proteins of the present invention, including for example Countercurrent Immuno-Electrophoresis, Radioimmunoassays, Radioimmunoprecipitations, Enzyme-Linked Immuno-Sorbent Assays (ELISA), Dot Blot assays, Western Blots, immunoprecipitation, Inhibition or Competition Assays, and sandwich assays (*see* U.S. Patent Nos. 4,376,110 and 4,486,530; *see also Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Following several clonal dilutions and reassays, a hybridoma producing antibodies reactive against the thymidine kinase mutant (or guanylate kinase enzyme or fusion protein) may be isolated.

Other techniques may also be utilized to construct monoclonal antibodies (*see* William D. Huse et al., "Generation of a Large Combinational Library of the Immunoglobulin Repertoire in Phage Lambda," *Science 246*:1275-1281, December 1989; *see also* L. Sastry et al., "Cloning of the Immunological Repertoire in *Escherichia coli* for Generation of Monoclonal Catalytic Antibodies: Construction of a Heavy Chain Variable Region-Specific

cDNA Library," *Proc. Natl. Acad. Sci. USA 86*:5728-5732, August 1989; *see also* Michelle Alting-Mees et al., "Monoclonal Antibody Expression Libraries: A Rapid Alternative to Hybridomas," *Strategies in Molecular Biology 3*:1-9, January 1990; these references describe a commercial system available from Stratacyte, La Jolla, California, which enables the production of antibodies through recombinant techniques). Briefly, mRNA is isolated from a B cell population, and utilized to create heavy and light chain immunoglobulin cDNA expression libraries in the kImmunoZap(H) and kImmunoZap(L) vectors. These vectors may be screened individually or co-expressed to form Fab fragments or antibodies (*see* Huse et al., *supra; see also* Sastry et al., *supra*). Positive plaques may subsequently be converted to a non-lytic plasmid which allows high level expression of monoclonal antibody fragments from *E. coli*.

Similarly, portions of antibodies may also be constructed utilizing recombinant DNA techniques to incorporate the variable regions of a gene which encodes a specifically binding antibody. Within one embodiment, the genes which encode the variable region from a hybridoma producing a monoclonal antibody of interest are amplified using nucleotide primers for the variable region. These primers may be synthesized by one of ordinary skill in the art, or may be purchased from commercially available sources. Stratacyte (La Jolla, Calif.) sells primers for mouse and human variable regions including, among others, primers for V_{Ha}, V_{Hb}, V_{Hc}, V_{Hd}, C_{HI}, V_L and C_L regions. These primers may be utilized to amplify heavy or light chain variable regions, which may then be inserted into vectors such as ImmunoZAP™ H or ImmunoZAP™ L (Stratacyte), respectively. These vectors may then be introduced into *E. coli* for expression. Utilizing these techniques, large amounts of a single-chain protein containing a fusion of the V_H and V_L domains may be produced (*see* Bird et al., *Science 242*:423-426, 1988). In addition, such techniques may be utilized to change a "murine" antibody to a "human" antibody, without altering the binding specificity of the antibody.

Once suitable antibodies have been obtained, they may be isolated or purified by many techniques well known to those of ordinary skill in the art (*see Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Suitable techniques include peptide or protein affinity columns, HPLC or RP-HPLC, purification on protein A or protein G columns, or any combination of these techniques.

LABELING OF ANTIBODIES

Anti-thymidine kinase, anti-guanylate kinase, or anti-fusion protein antibodies which are described above may be labeled with a variety of molecules, including for example,

fluorescent molecules, toxins, and radionuclides. Representative examples of fluorescent molecules include fluorescein, phycoerythrin, rodamine, Texas red and luciferase. Representative examples of toxins include ricin, abrin diphtheria toxin, cholera toxin, gelonin, pokeweed antiviral protein, tritin, Shigella toxin, and Pseudomonas exotoxin A. Representative examples of radionuclides include Cu-64, Ga-67, Ga-68, Zr-89, Ru-97, Tc-99m, Rh-105, Pd-109, In-111, I-123, I-125, I-131, Re-186, Re-188, Au-198, Au-199, Pb-203, At-211, Pb-212 and Bi-212. In addition, the antibodies described above may also be labeled or conjugated to one partner of a ligand binding pair. Representative examples include avidin-biotin, and riboflavin-riboflavin binding protein.

Methods for conjugating or labeling the anti-thymidine kinase, anti-guanylate kinase, or anti-fusion protein antibodies discussed above with the representative labels set forth above may be readily accomplished by one of ordinary skill in the art (*see* Trichothecene Antibody Conjugate, U.S. Patent No. 4,744,981; Antibody Conjugate, U.S. Patent No. 5,106,951; Fluorogenic Materials and Labeling Techniques, U.S. Patent No. 4,018,884; Metal Radionuclide Labeled Proteins for Diagnosis and Therapy, U.S. Patent No. 4,897,255; and Metal Radionuclide Chelating Compounds for Improved Chelation Kinetics, U.S. Patent No. 4,988,496; *see also* Inman, *Methods In Enzymology*, Vol. 34, *Affinity Techniques, Enzyme Purification: Part B*, Jakoby and Wilchek (eds.), Academic Press, New York, p. 30, 1974; *see also* Wilchek and Bayer, "The Avidin-Biotin Complex in Bioanalytical Applications," *Anal. Biochem. 171*:1-32, 1988).

PHARMACEUTICAL COMPOSITIONS

As noted above, the present invention also provides a variety of pharmaceutical compositions (or medicaments), comprising one of the above-described thymidine kinase mutants, guanylate kinases, or fusion proteins (*e.g.* either the nucleic acid molecule, vector, or protein), along with a pharmaceutically or physiologically acceptable carrier, excipients or diluents. Generally, such carriers should be nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the therapeutic agent with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with nonspecific serum albumin are exemplary appropriate diluents.

In addition, the pharmaceutical compositions of the present invention may be prepared for administration by a variety of different routes, including for example

intraarticularly, intracranially, intradermally, intramuscularly, intraocularly, intraperitoneally, intrathecally, intravenously, subcutaneously or even directly into a tumor (for example, by stereotaxic injection). In addition, pharmaceutical compositions of the present invention may be placed within containers, along with packaging material which provides instructions regarding the use of such pharmaceutical compositions. Generally, such instructions will include a tangible expression describing the reagent concentration, as well as within certain embodiments, relative amounts of excipient ingredients or diluents (*e.g.*, water, saline or PBS) which may be necessary to reconstitute the pharmaceutical composition.

METHODS

The present invention also provides methods for inhibiting a pathogenic agent in a warm-blooded animal, comprising administering to the warm-blood animal a vector (*e.g.*, expression vector, viral vector, or viral particle containing a vector), as described above, such that the pathogenic agent is inhibited. Representative examples of pathogenic agents include autoimmune cells, tumor cells, cells which do not express or inappropriately express a particular gene, and cells infected with bacteria, viruses, or other intracellular parasites. As will be evident to one of skill in the art, the amount and frequency of administration will depend, of course, on such factors as the nature and severity of the indication being treated, the desired response, the condition of the patient, and so forth. Typically, the compositions may be administered by a variety of techniques, including for example intraarticularly, intracranially, intradermally, intramuscularly, intraocularly, intraperitoneally, intrathecally, intravenously, subcutaneously or even directly into a tumor (for example, by stereotaxic injection).

Within certain embodiments of the invention, the vectors which contain or express the nucleic acid molecules which encode thymidine kinase (and/or guanylate kinase) or fusion protein described above, or even the nucleic acid molecules themselves may be administered by a variety of alternative techniques, including for example administration of asialoosomucoid (ASOR) conjugated with poly (L-lysine) DNA complexes (Cristano et al., *PNAS* 92122-92126, 1993), DNA linked to killed adenovirus (Michael et al., *J. Biol. Chem.* *268*(10):6866-6869, 1993; and Curiel et al., *Hum. Gene Ther.* *3*(2):147-154, 1992), cytofectin-mediated introduction (DMRIE-DOPE, Vical, Calif.), direct DNA injection (Acsadi et al., *Nature* *352*:815-818, 1991); DNA ligand (Wu et al., *J. of Biol. Chem.* *264*:16985-16987, 1989); lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA* *84*:7413-7417, 1989); liposomes (Pickering et al., *Circ.* *89*(1):13-21, 1994; and Wang et al., *PNAS* *84*:7851-7855, 1987); microprojectile bombardment (Williams et al., *PNAS* *88*:2726-2730, 1991); retrotransposons,

transferrin-DNA complexes (Zenke), and direct delivery of nucleic acids which encode the enzyme itself either alone (Vile and Hart, *Cancer Res. 53*: 3860-3864, 1993), or utilizing PEG-nucleic acid complexes.

Within one aspect of the invention, methods are provided for inhibiting a tumor or cancer in a warm-blooded animal, comprising administering to the warm-blooded animal one of the vectors described above (or nucleic acid molecules which encode thymidine kinase mutants, guanylate kinase enzymes, or fusion proteins of the present invention), such that the tumor or cancer is inhibited. Within one embodiment, selected cells may be removed from a warm-blooded animal, one or more of the vectors described above introduced into the removed cells, and the cells reintroduced into the same or another warm-blooded animal. Within other embodiments, vectors or nucleic acid molecules which encode thymidine kinase (or mutants as described herein) or guanylate kinase or fusion protein may be separately administered or introduced. Within a further embodiment, such methods further comprise the step of administering a nucleoside analogue. Representative examples of such nucleoside analogues include ganciclovir, acyclovir, trifluorothymidine, 1-[2-deoxy, 2-fluoro, beta-D-arabino furanosyl]-5-iodouracil, ara-A, araT 1-beta-D-arabinofuranoxyl thymine, 5-ethyl-2'-deoxyuridine, 5-iodo-5'-amino-2,5'-dideoxyuridine, idoxuridine, AZT, AIU (5-iodo-5' amino 2', 5'-dideoxyuridine), dideoxycytidine and AraC. Briefly, utilizing such methods, a wide variety of tumors (both benign and malignant) may be treated. Representative examples of such tumors include solid tumors such as lung carcinomas, renal cell carcinomas, breast carcinomas, colorectal carcinomas and melanomas, as well as diffuse cancers such a leukemias and lymphomas.

Within other aspects of the present invention, methods are provided for treating a variety of diseases wherein a subset of cells may be characterized as "diseased" or altered, utilizing the above-described nucleic acid molecules or vectors. Representative examples of such diseases include hyperkeratosis (psoriasis), prostate hypertrophy, hyperthyroidism, a wide variety of endocrinopathies, autoimmune diseases (due to autoimmune reactive cells such as certain subsets of T cells), allergies (*e.g.*, by modulating the activity of IgE expressing cells responsible for an allergic response), restenosis (*e.g.*, by killing cells which are responsible for the ingrowth and/or clogging of a blood vessel), a wide array of viral diseases such as AIDS (HIV), hepatitis (HCV or HBV), and intracellular parasitic diseases. Within other embodiments of the invention, methods are provided for inhibiting the growth of or destroying cells which are not traditionally associated with a disease. For example, within certain embodiments it may be desirable to administer a vector (or nucleic acid molecule

alone) which inhibits or destroys fat cells in order to initiate weight loss in an animal, or to destroy hair follicles (as a depilatory reagent).

Within yet other aspects, vectors which contain or express the nucleic acid molecules encoding thymidine kinase mutants and/or guanylate kinase, or fusion protein (or the nucleic acid molecules themselves) may be utilized to correct aberrant expression of a gene within a cell, or to replace a specific gene which is defective in proper expression. Representative examples of such diseases include Adenosine Deaminase Deficiency, Alzheimer's Disease (*see, for example*, Goat et al., *Nature 349*:704, 1991; Sherrington et al., *Nature 375*:754, 1995; Levy-Labad et al., *Science 269*:973, 1995), Cystic Fibrosis, as well as, for example, diseases such as Hemophilia.

Within other aspects of the present invention, methods are provided for utilizing the thymidine kinase mutants or fusion proteins described above, as a negative-selection marker gene (*see e.g.*, Czako and Marton, *Plant Physiol. 104*:1067-1071, 1994) in prokaryotic cells, eukaryotic cells, plants (Czako and Morton, Plant Physiol. 104:1067-1071, 1994), parasites (*e.g.*, Trypanosomes) or viruses. Alternatively, such mutants may be utilized as a conditionally lethal marker for homologous recombination (Mansour et al., *Nature 336*:348-352, 1988). A representative example is set forth in more detail below as Example 6.

Within other aspects of the present invention, methods are provided for noninvasive monitoring of gene therapy using thymidine kinase mutants and fusion proteins having thymidine kinase and guanylate kinase activities. Methods have been developed for the noninvasive imaging of HSV-1 thymidine kinase gene expression using a clinical gamma camera and by single-photon emission tomography with radiolabeled thymidine kinase substrate (see, for example, Tjuvajev et al., *Cancer Res. 55*:6126-6132, 1995; Tjuvajev et al., *Cancer Res. 56*:4087-4095, 1996). The basic approach is to administer a labeled anti-viral drug that is selectively phosphorylated by HSV-1 thymidine kinase and to monitor progress of therapy using standard scanning methods for human diagnosis. Suitable radiolabeled anti-viral drugs that are substrates for HSV-1 thymidine kinase, such as IVFRU, are well-known to those of skill in the art. See, for example, Wiebe et al., *Q. J. Nucl. Med. 41*:79-89 (1997), which contains a discussion of imaging with radiolabeled nucleoside substrates for HSV-1 TK that is incorporated by reference. The mutant thymidine kinases and fusion proteins of the present invention that have enhanced thymidine kinase activity provide a means to increase the sensitivity of such noninvasive monitoring.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

EXAMPLE 1

CONSTRUCTION OF TK MUTANTS CONTAINING MUTATIONS AT CODONS 165-175 UTILIZING A 20% RANDOM LIBRARY

Example 1 describes the construction of TK mutants containing mutations at codons 165 to 175, utilizing a 20% random library. A schematic outline which depicts the strategy utilized in this example is set forth in Figure 1.

A. Generation of TK Mutants

1. Generation of Oligonucleotides

A 52-mer oligonucleotide with a wild-type *tk* sequence (SEQUENCE ID. No. 2) and a 56-mer that contained degenerate nucleotides spanning from codon 165 through 175 (SEQUENCE ID. No. 3) of the *tk* gene (Figure 23 discloses nucleotides in the open reading frame of HSVTK-1 [SEQUENCE ID NO. 1]), (where N = 80% wild-type nucleotides and a 20% mixture of the other three at each position) were synthesized by Operon Technologies (San Pablo, CA). Both oligomers were complementary to each other along 12 bases at their 3'-ends.

5'-TG GGA GCT CAC ATG CCC CGC CCC CGG CCC TCA CCC TCA TCT TCG ATC GCC AT-3' (SEQUENCE ID No. 2)
5'-ATG AGG TAC CGN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNA TGG CGA TCG AA-3' (SEQUENCE ID No. 3)

For the construction of pKTPD described below, two additional oligonucleotides were synthesized by Operon Technologies using phosphoramide chemistry. These oligonucleotides were:

5'-CCC CTC GAG CGC GGT AC-3' (SEQUENCE ID No. 4)
5'-CGC GCT CGA GGG GAG CT-3' (SEQUENCE ID No. 5)

2. Generation of Random Sequence-Containing Libraries

a. Construction of Vectors pMDC and pMCC

Chimeric vectors pMDC (which produces an inactive TK gene product) and pMCC (which produces wild-type TK) were produced from plasmids pHETK1 and pHETK2 essentially as described below. Briefly, plasmids pHETK1 and pHETK2 (Waldman et al., *J.*

Biol. Chem. 258:11571-11575, 1983) are expression vectors that contain a HSV-1 *tk* structural gene, and are derivatives of pBR322. Restriction maps of pHETK1 and pHETK2 can be found in Waldman *et al, J. Biol. Chem. 258*:11571-11575, 1983, which describes the construction of these plasmids. Plasmid pHETK2 contains λP_L and λP_R promoters, ampR, and the c1857 temperature-sensitive repressor, whereas pHETK1 contains all the above except the λP_L promoter. Plasmids pHETK1 and pHETK2 were obtained from Dr. William Summers (School of Medicine, Yale University, New Haven).

In order to construct pMDC and pMCC, a dummy vector, designated pKTPD was first constructed as described by Dube et al. in *Biochem. 30*:11760-11767, 1991. Briefly, oligonucleotides SEQUENCE ID Nos. 4 and 5 (20 pmol of each) were first phosphorylated and then annealed to form a double-stranded oligonucleotide with *Kpn*I- and *Sst*I-compatible ends and with an internal *Xho*I site. In addition, pHETK2 was digested with *Sst*I and *Kpn*I restriction endonucleases, and the large fragment isolated by agarose gel electrophoresis and subsequent electroelution. Two picomoles of the large fragment was ligated with 6 pmol of the double-stranded oligonucleotide. The resultant double-stranded circular DNA product (designated "pKTPD") was used to transform competent *E. coli* KY895 cells. *E. coli* KY895 is a TK-deficient strain (K12 *tdk*$^-$, F$^-$, *ilv* 276) obtained from William Summers, Yale University, New Haven, CT. Clones containing the recombinant plasmid pKTPD grow on LB plates containing 50 μg/mL carbenicillin. The presence of recombinant plasmid DNA was verified by the cleavage at the *Xho*I site. The inability of pKTPD to support the growth of *E. coli* KY895 in the thymidine kinase selection medium indicates that it does not produce a functional thymidine kinase.

pHETK1 and pKTPD were then utilized to construct a new chimeric dummy vector, designated pMDC. Briefly, upon digestion with *Sph*I and *Pvu*II pHETK1 is cut into two fragments. The larger fragment contains ampR, cI857, λP_R sequences, and part of the *tk* gene spanning from the BamHI to the SphI site. The smaller fragment contains the remainder of the *tk* gene from *Sph*I to *Pvu*II. Similarly, pKTPD upon digestion with the same two enzymes is cut into one larger and one smaller fragment. The smaller *SphI*/*Pvu*II fragment of pKTPD contains a dummy or inactive sequence within the *Kpn*I and *Sac*I sites of the *tk* gene. Ligation of the larger fragment from pHETK1 with the smaller fragment of pKTPD results in a chimeric vector, pMDC, that produces an inactive *tk* gene product.

Another chimeric vector, pMCC, containing the wild-type *tk* gene was similarly constructed by ligating the larger fragment from pHETK1 with the smaller fragment of pHETK2. As noted above, PMCC produces active wild-type TK.

b. Generation of a Library

A library containing 20% random nucleotide sequences was constructed as follows. Briefly, a 52-mer oligo containing wild-type sequences (SEQUENCE ID No. 2) was hybridized to a 56-mer oligo which contained degenerate sequences spanning codons 165 through 175 (Sequence ID No 3).

The hybrid was extended with the Klenow fragment of *E. coli* DNA polymerase I to produce a complete double-stranded DNA product. This strategy was implemented in order to avoid synthesizing a long random nucleotide containing SEQUENCE ID No. 3, since the locations of *Kpn*I and *Sac*I sites (insertion sites) in the vector require a long cassette. The Klenow fragment generated double-stranded DNA was then subjected to polymerase chain reaction amplification by using two synthetic primers: the first primer, a: 5'-TGG GAG CTC ACA TGC CCC GCC-3' (SEQUENCE ID No. 6) corresponds to the 21-base sequence of 5' terminus of oligo SEQUENCE ID No. 2. The second primer, b: 5'-ATG AGG TAC CG-3' (SEQUENCE ID No. 7) corresponds to the 11-base sequence of 5' terminus of oligo SEQUENCE ID No. 3. The polymerase chain reaction amplification reactions contained 20 mM Tris-HCl (pH 8.3), 25 mM KCl, 1.5 mM $MgCl_2$, and 0.05% Tween 20, 0.1 mg/ml BSA, 50 μM each of the four deoxynucleoside triphosphates, 20 pmol of primer "a," 40 pmol of primer "b," approximately 1 pmol of the extended double-stranded oligonucleotide as template, and 2 units of *Taq* polymerase (Cetus) in 100-μl final reaction volumes. Each mixture was overlaid with mineral oil and subjected to 30 rounds of temperature cycling: 94°C for 1 minute, 34°C for 2 minutes, and 72°C for 7 minutes.

Low molecular weight components and excess primers were removed from the polymerase chain reaction-amplified product by centrifugation with a Centricon 30 ultrafiltration unit, and the amplified DNA was digested with *Kpn*I and *Sac*I. The digested double-stranded oligonucleotide containing the random sequence was again purified by a Centricon 30 unit, and ligated to the *Kpn*I/*Sac*I digested large fragment of pMDC at 10:1 molar ratio in the presence of 1 mM ATP and 1 unit of T4 DNA ligase (BRL) in a volume of 10 μl. Incubation was for 18 hours at 14°C and the reaction was terminated by phenol-$CHCl_3$ extraction followed by ethanol precipitation.

c. Selection of TK Mutants

The precipitate described above was dried and dissolved in 10 μl of water, and used to transform competent *E. coli* KY895 by electroporation. One μl of ligated product was mixed with 50 μl of competent cells and electroporated at 2 KV, 25 μF, and 400 Ohms with a

Gene-pulser electroporator (Bio-Rad). After the pulse, 1 ml of SOC medium (2% Bacto-tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added, followed by incubation at 37°C for 1.5 hours with continuous agitation. An aliquot of each transformation solution was spread onto LB-agar medium containing 50 μg/ml of carbenicillin to determine total number of transformants. Selection for active TK clones was performed on TK selection medium that contained 50 μg/ml of carbenicillin. 10 μ g/ml of 5' fluorodeoxyuridine, 2 μg/ml of thymidine, 20 μg/ml of uridine. 2% BBL peptone, 0.5% NaCl, 0.2% glucose, and 0.8% Gel-Rite (Scott Laboratories, Inc., Carson, CA) (Fig. 1). Colonies on carbenicillin medium were incubated at 37°C for 14-16 hours, whereas inoculated TK selection medium was incubated at 37°C for 24 hours.

From a total of 53,000 transformants that grew on carbenicillin medium, 190 were able to complement *E. coli* KY 895 for TK function.

EXAMPLE 2

CONSTRUCTION OF TK MUTANTS CONTAINING MUTATIONS AT CODONS 165-175 UTILIZING A 100% RANDOM LIBRARY

Example 2 describes the construction of TK mutants containing mutations at codons 165-175 utilizing a 100% random library. The strategy which was utilized for this example is similar to that described in Example 1 above.

A. Generation of TK Mutants

1. Generation of Oligonucleotides

A 52-mer 5'-d(TG GGA GCT CAC ATG CCC CGC CCC CGG CCC TCA CCC TCA TCT TCG ATC GCC AT)-3' (SEQUENCE ID No. 8) with a wild-type *tk* sequence and *Kpn* I site at the 5' end was synthesized by Operon Technologies (San Pablo, CA). In addition, a 56-mer containing random nucleotides corresponding to HSV-1 *tk* codons 165-175 and containing a *Sac* I site at the 3' end 5' -d(ATG AGG TAC CGN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNA TGG CGA TCG AA)-3' (SEQUENCE ID No. 3), where N = equimolar concentrations of G, A, T, or C, was also synthesized. The oligonucleotides were separated by electrophoresis through a 20% denaturing polyacrylamide gel, followed by purification on a reverse-phase mini column (Glen Research, Sterling, VA).

2. Generation of a 100% Random Sequence - Containing Library

The 52-mer corresponding to the wild-type HSV-1 *tk* sequence was hybridized with the 56-mer containing random nucleotides. The hybrid was then extended with the Klenow fragment of DNA polymerase I, PCR amplified, and ligated into pMDC essentially as described above in Example 1.

3. Selection of TK^+ Mutants

Functional TK mutants were identified by colony formation on TK-selection medium based on their ability to phosphorylate dT essentially as described below. Briefly, the ligated product was introduced into tk^- *E. coli* strain KY895. The total number of transformants was determined by plating on LB agar containing 50 μg of carbenicillin per mL and the number of transformants that produced catalytically active thymidine kinase was determined by plating on TK-selection medium [2% BBL peptone, 0.5% NaCl, 0.2% glucose, 0.8% Gel-Rite (Scott Laboratories, Carson, CA)], 50 μg 1 mL of carbenicillin, 10 μg/mL of fluorodeoxyuridine, 2 μg/mL of dT, and 20 μ g/mL of uridine.

Two million (2×10^6) transformants were screened from the 100% random library, of which 1540 formed colonies on the TK-selection medium.

B. Selection of AZT-Sensitive Mutants

A subset of 690 mutants from the 100% random library (TKI) and 190 mutants from the 20% degenerate library (TKF) (described above in Example 1) were subjected to secondary negative selection on medium containing AZT in order to identify mutants that exhibited enhanced phosphorylation of AZT. This screen is based upon the premise that mutants with increased ability to phosphorylate AZT relative to dT would be unable to form colonies on the AZT-selection medium. In particular, the product, AZT monophosphate would be further phosphorylated by the host cell's nonspecific nucleotide kinases, or possibly by the mutant TK, incorporated into bacterial DNA by host DNA polymerases, terminate DNA synthesis, and thus prevent replication of the host chromosome.

Briefly, the TK mutants were first grown as individual colonies on TK-selection medium (1.0 μg/mL of dT), and then replica plated onto AZT-selection medium (0.05 μg/mL of AZT, 1.0 μg/mL of dT). All other components in the AZT-selection medium were the same as the TK-selection medium. Those TK mutants which failed to grow on the

AZT-selection medium were selected and retested for growth on both TK- and AZT-selection media separately.

Of the 880 primary selectants that were screened, only two mutants, TKF 105 (SEQ ID NOs. 55 and 56) (from the 20% library) and TKI 208 (SEQ ID NOS. 22 and 23) (from the 100% library), formed colonies on the TK-selection medium at an efficiency similar to that of *E. coli* harboring the wild-type plasmid but not on the AZT-selection medium (Figure 2).

The nucleotide and deduced amino acid sequences of TKF 105 (SEQ ID NOs. 55 and 56) and TKI 208 (SEQ ID NOs. 22 and 23) are presented in Figure 3. Both mutants contain a single amino acid substitution at the same position: Leu-170 was changed to Ile in TKF 105 (SEQ ID NOs. 55 and 56) and to Val in TKI 208 (SEQ ID NOS. 22 and 23). No other substitutions were observed in the surrounding 220 nucleotides.

To ensure that the difference between TKF 105 (SEQ ID NOs. 55 and 56) and TKI 208 (SEQ ID NOS. 22 and 23) was not due to differential expression of TK in *E. coli* harboring mutant and wild-type plasmids, Western blots of extracts from cells containing either TKI 208 (SEQ ID NOS. 22 and 23) or wild-type plasmids were compared. No significant difference was observed in the amount or electrophoretic mobility of immunoreactive staining protein. Also, the rate of dT phosphorylation per mg of protein was determined, and found to be similar in extracts of *E. coli* harboring TKI 208 (SEQ ID NOS. 22 and 23), TKF 105 (SEQ ID NOs. 55 and 56), and wild-type plasmids.

In order to show that the lack of growth of these two mutants on AZT-selection medium was due to enhanced phosphorylation of AZT, the following experiments were conducted.

1. Rate of [^{3}H]AZT Uptake

First, the rate of [^{3}H]AZT uptake relative to [^{3}H]dT into *E. coli* harboring wild-type and mutant plasmids was determined. These studies indicated that *E. coli* harboring the AZT-sensitive mutants, TKF 105 (SEQ ID NOs. 55 and 56) and TKI 208 (SEQ ID NOS. 22 and 23), exhibited a 4-fold increase in the ratio of AZT to dT uptake, as compared to *E. coli* with the wild-type plasmid.

2. Affinity Purification of TK

Purification of wild-type and mutant TKs was performed by affinity chromatography on CH-Sepharose 4B (Pharmacia) coupled to *p*-aminophenylthymidine

3'-phosphate. Briefly, crude bacterial extract was passed three times through a 7-mL bed-volume affinity column. The column was then washed sequentially using 30 mL each of buffer A [0.1 M Tris HC1, pH 7.5/5 mM dithiothreitol (DTT)/10% glycerol], buffer B (0.1M Tris-HC1, pH 7.5/0.5 M KC1/5 mM DTT/10% glycerol), and buffer A. TK was eluted using a 60-mL linear gradient of 0-600 μM dT in buffer C (0.3 M Tris HCl, pH 7.4/50 mM KCl/10% glycerol). Active fractions were pooled and dialyzed against three changes each of 2 liters of 50 mM Tris-HC1, pH 7.4/5 mM DTT/10% glycerol. Except in the final dialysis, all the above buffers contained 50 μg/mL of aprotinin and 2 μg/mL each of pepstatin and leupeptin.

3. Kinetics of AZT Phosphorylation

Secondly, the kinetics of AZT phosphorylation by the two mutants was determined. Briefly, reactions were carried out in a final volume of 100 μl containing 50 mM Tris-HCl (pH 7.5), 5 mM ATP. 4 mM $MgCl_2$, 2.5 mM DTT, 12 mM KCl, 0.18 mg/mL of bovine serum albumin, 5% glycerol, 0.08 μCi of [^{3}H]AZT (Sigma), various concentrations of unlabeled AZT (0-4.0 μM), and purified enzymes (4 and 1.2 units, respectively, for wild-type and TKI 208 (SEQ ID NOS. 22 and 23)). (One unit of enzyme is defined as that amount that can phosphorylate 1.0 pmol of dT to TMP in 1 minute under the conditions described above.) Incubation was at 34°C ± 1°C for 10 minutes, and reactions were stopped by adding 1.0 mM unlabeled dT and cooling on ice. Half of the reaction mixtures were pipetted onto a DEAE-cellulose disc (25 mm), dipped in distilled water (1 minute), followed by four washes in absolute ethanol. The amount of radioactivity adsorbed to the disc was determined by scintillation spectroscopy. K_m and V_{max} values were determined by using the Cleland SUBIN program (Cleland, *Methods Enz. 63*:103-138, 1979). The values for k_{cat} were calculated using the equation $V_{max} = k_{cat}[E]_o$, where $[E]_o$ = total enzyme concentration. TK assays wherein phosphorylation of dT was measured were carried out in a final volume of 50 μl using 0.3 μCi ([3H-*methyl*]dT: 87 Ci/mmol: Amersham), various concentrations of unlabeled dT (0-4.0 μM), and 1.1 and 0.5 units of TK for the wild-type and TKI 208 (SEQ ID NOS. 22 and 23), respectively. All other components in the reaction mixtures and the incubation conditions were as described above for phosphorylation of AZT.

As shown below in Table I, the AZT-sensitive variant TKI 208 (SEQ ID NOS. 22 and 23) exhibits a lower K_m (4.4 μM) compared to that of the wild-type (8.5 μM). By comparing the k_{cat}/K_m between the two substrates (AZT vs. dT), it can be seen that TKI 208

(SEQ ID NOS. 22 and 23) selectively phosphorylates AZT 2.3-fold more efficiently than dT. Similar preliminary experiments with purified TKF 105 (SEQ ID NOs. 55 and 56) TK also showed lower K_m (3.7 μM) for AZT, but similar values for k_{cat}/K_m compared to the wild-type.

TABLE I

ABILITY OF WILD-TYPE AND TKI 208 TKS TO PHOSPHORYLATE AZT AND DT

Phosphorylation		K_m,μM	k_{cat},s^{-1}	k_{cat}/K_m s^{-1},M^{-1}	k_{cat}/K_m(AZT) k_{cat}/K_m(dT)
AZT					
	Wildtype	8.46 ± 1.3	3.6×10^{-2}	4.2×10^{3}	1.7×10^{-3}
	TKI 208	4.40 ± 0.43*	3.0×10^{-2}	6.5×10^{3}	4.0×10^{-3}
dT					
	Wildtype	0.475 ± 0.10	1.21	2.5×10^{6}	
	TKI 208	0.35 ± 0.008	0.56	1.57×10^{6}	

C. Thermostability Analysis of Mutant TKs

Mutants were analyzed for thermostability essentially as described below. Briefly, 25 μg of each extract were preincubated in 0.3 mL of 28 mM Tris-HCl, pH 7.5 containing 0.28 mg/mL of bovine serum albumin, 28 μg/mL of aprotinin, 2 μ g/mL (each) of pepstatin and leupeptin, at 42°C for 0.5, 10, 20, 30, or 40 minutes. At each time point 30-μl (2.5 μg) aliquots were assayed for residual TK activity in a total reaction volume of 50 μl containing 50 mM Tris-HCl (pH 7.5), 5 mM ATP, 4 mM $MgCl_2$, 2.5 mM DTT, 12 mM KCl, 0.18 mg/mL of bovine serum albumin, 5% glycerol, and 1 μM [^{3}H-*methyl*]dT (60×10^3 dpm/pmol). Incubation was at 34°C for 10 minutes. The reaction was stopped by cooling on ice, and 25 μl was pipetted onto a DEAE-cellulose disc. Wash and assay conditions for the discs were performed as described for the AZT assay above.

Assay results of unfractionated extracts of TKF 2 (SEQ ID NOs. 57 and 58), TKF 56 (SEQ ID NO. 167), TKF 75 (SEQ ID NO. 166), TKF 446 (SEQ ID NO. 168) and wild-type TK are shown in Figures 4A-4D. One of the mutants, TKF 2 (SEQ ID NOs. 57 and 58), was more thermostable at 42°C than any of the other mutants, or than the wild-type. Except for TKF 2 (SEQ ID NOs. 57 and 58), all of the mutants tested, including the wild-type, had ratios of residual activity after preincubation at 42°C compared to 34°C of 0.05-0.30: TKF 2 (SEQ ID NOs. 57 and 58) had a ratio of 0.7. TKF 2 (SEQ ID NOs. 57 and 58) contains three amino acid substitutions: Pro-165 → His, Ala-167 → Ser, and Ala-174 → Val

(Figure 3). TKF 75 (SEQ ID NO. 166) contained an Ala-167 → Ser substitution, TKF 56 (SEQ ID NO. 167) a Ala-174 → Val, and TKI 440 (SEQ ID NO. 168) a Pro-165 → Ala substitution. The thermolability of mutants TKF 56 (SEQ ID NO. 167) and TKF 75 (SEQ ID NO. 166) with Ala-174 → Val and Ala-167 → Ser substitutions, respectively, was similar to that of the wild-type. Both lost >80% of their activity after incubation for 5 minutes at 42°C. TKF 440 (SEQ ID NO. 168) with a Pro-165 → Ala is more stable, but not as stable as TKF 2 (SEQ ID NOs. 57 and 58), the triple mutant.

Two types of experiments were carried out to verify the thermostability of TKF 2 (SEQ ID NOs. 57 and 58). First, TK protein from TKF 2 (SEQ ID NOs. 57 and 58) and the wild-type plasmid harboring *E. coli* were purified to near homogeneity by affinity chromatography, and assayed as described above. As before, loss of activity is less in TKF 2 (SEQ ID NOs. 57 and 58) than in the wild-type after preincubation at 42°C (Figure 4E).

Secondly, *tk* genes from TKF 2 (SEQ ID NOs. 57 and 58) and wild-type TK were transferred into a vector with a promoter for T3 RNA polymerase. More specifically, the full-length *Bgl* II-*Pvu* I fragments of *tk* genes from wild-type and TKF 2 (SEQ ID NOs. 57 and 58) plasmids were isolated and subcloned into the pBluescript SK^+ (Stratagene) vector between the *Spe* I and *Eco*RI sites with the use of synthetic linkers. *In vitro* transcription using the T3 promoter was carried out using the Promega transcription system. *In vitro* translation was carried out using a reticulocyte lysate system (Promega) following the supplier's protocol. The loss of TK activity of the *in vitro* synthesized proteins from the wild-type and TKF 2 (SEQ ID NOs. 57 and 58) *tk* genes as a function of preincubation at 42°C is shown in Figure 5. The protein encoded by TKF 2 (SEQ ID NOs. 57 and 58) lost <10% of its activity after preincubation for 45 minutes. In contrast, the protein encoded by the wild-type gene lost >80% of its initial activity. The degree of thermostability exhibited by the *in vitro* synthesized TKF 2 (SEQ ID NOs. 57 and 58) was similar to or greater than that of crude extracts harboring the original TKF 2 (SEQ ID NOs. 57 and 58) plasmid. For SDS/PAGE analysis, the translated products were labeled with [^{35}S]methionine.

An autoradiograph of the labeled proteins after SDS/PAGE is shown in Figure 6. The arrow indicates the expected size of translated TKs as judged by molecular mass standards (Bio-Rad). From this autoradiograph it is evident that the translation products migrate as double bands, one of which corresponds to a protein of 43 kDa, which is in accord with the reported size of HSV-1 TK expressed in *E. coli*. The second band could be due to the proteolytic degradation of a 32-residue fragment at the amino-terminal end, which does not detectably alter TK activity of the HSV-1 TK.

EXAMPLE 3

CONSTRUCTION AND ANALYSIS OF TK MUTANTS WITH MUTATIONS AT CODONS 155, AND 161 TO 165 UTILIZING A 20% RANDOM LIBRARY

This example describes the construction and analysis of TK mutants which are mutagenized at codons 155, and 161 through 165. Bacterial strains and materials which were utilized within this example are set forth below.

Bacterial Strains. E. coli strain KY895 (F^-, *tdk*$^-$, 1-*ilv*), originally described by Igarashi et al. (*Genetics 57*:643-654, 1967), was used in the genetic complementation assays for thymidine kinase activity. *E. coli* strain NM522 (F' *lacI*^{q} Δ (*lacZ*)M15 *pro*AB/*sup*E *thi* Δ (*lac* proAB)Δ(*hsd*MS-*mcr*B)5(r_k^-McrB$^-$)) (NEB, Beverly, MA) was used as a recipient in all subcloning experiments. Helper phage VCM13 (Stratagene, La Jolla, CA) was used in the production of single-stranded phage for sequencing.

Materials. L-[^{35}S]Methionine/cysteine (specific activity, 1140 Ci/mmol) for protein synthesis determination and [*methyl*-^{3}H] thymidine (specific activity, 87 Ci/mmol) were purchased from Amersham. Other radioisotopes [[*side chain*-2-^{3}H] acyclovir (specific activity, 28.6 Ci/mmol) and [5-^{3}H]-deoxycytidine (specific activity, 29 Ci/mmol)] were purchased from Du Pont-New England Nuclear (Boston, MA), and [8-^{3}H] ganciclovir (specific activity, 22 Ci/mmol) and [*methyl*-^{3}H]-3'-azido-3' deoxythymidine (specific activity, 14 Ci/mmol) were from Moravek (Brea, CA). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (NEB). Promega (Madison, WI) was the source of the *in vitro* transcription and translation reagents except for the cap analog, ^{7}m(5')Gppp(5')G, which was purchased from NEB. Oligonucleotides used for sequencing and polymerase chain reaction amplifications were obtained from Operon (Alameda, CA). Other chemicals were purchased from Sigma (St. Louis, MO) except where designated.

A. Generation of TK Mutants

1. Generation of Oligonucleotides

Two oligonucleotides were synthesized by American Synthesis, Inc. (Pleasanton, CA): MB 110 (70mer) 5'-TGGGAGCTCA CATGCCCCGC CC[CCG]GCCCT

CACCCTCATC [TTCGACCGCC ATCCC]ATCGC CGCCCTCCTG-3' (SEQUENCE ID No. 9), and MB111 (38mer) 5'-ATGAGGTACC GCGCAGCTGG GTAGCACAGG AGGGCGGC-3' (SEQUENCE ID No. 10). Within these oligonucleotides, nucleotides in brackets where synthesized as 80% wild-type nucleotide, and 20% the other three nucleotides.

At the 5' end of MB 110 is a *Sac*I restriction site and, at the 5' end of MB111, a *Kpn*I site. These restriction sites were utilized at a later step after second-strand synthesis occurred. Furthermore, as an internal control, a *Pvu*II site was introduced (silent change) in MB111 in order to allow confirmation of random sequence insertion prior to sequencing. Twelve nucleotides at the 3' ends of each oligonucleotide are complementary to allow for hybridization of the two strands to each other. Each oligonucleotide was subjected to electrophoresis on a 20% acrylamide-urea gel and visualized by UV shadowing on a PEI-cellulose TLC plate (Baker, Phillipsburg, NJ), the portion of the gel containing the correct sized oligonucleotide was excised, and the oligonucleotide was eluted from the gel in 0.5M NH4Ac/10mM $MgOAc_2$ overnight at 37°C. The eluted oligonucleotide was then ethanol-precipitated and resuspended in H2O. An OD_{260} measurement was taken, and the extinction coefficient for each oligo was used to determine the concentration.

Equimolar amounts of MB110 and MB111 (25pmol) were annealed in a small volume (20μl) in 1 x annealing buffer (10 x annealing buffer = 70mM Tris (pH 7.5)/60mM $MgC1_2$/200mM NaCl) for 5 minutes at 95°C, then moved to 65°C for 20 minutes, followed by slow cooling to room temperature. To the annealed oligonucleotides (20μl) were added 2μl of 10 x annealing buffer, 2.8μl of 10mM dNTPs, 0.8μl of 0.1M of dithiothreitol (DTT), 2.4μl of DNA polymerase I Klenow fragment (5 units/μl), and H_2O to bring the volume to 40μL. The mixture was placed at 37°C for 30 minutes, at 65°C for 10 minutes, and finally at room temperature for 10 minutes. Verification of fully extended radioactive oligonucleotides was accomplished by subjecting the samples to denaturing acrylamide gel electrophoresis and autoradiography. Amplification of the extended products was performed using the polymerase chain reaction with Taq polymerase (Stratagene). The 100 uL reactions contained 20mM Tris (pH 8.3)/25mM KCl/1.5mM $MgCl_2$/0.05% Tween 20)/0.1mg/mL BSA/50μM of each of the four deoxynucleoside triphosphates (dNTPs)/22pmol PCR primer 1/20pmol PCR primer 2/2 units of Taq polymerase and 6 pmol of the extended random oligonucleotide; Primer 1 = 5' TGGGAGCTCACATGCCCCGCC-3' (SEQUENCE ID No. 6) and primer 2 = 5'-ATGAGGTACCG-3' (SEQUENCE ID No. 7). One drop of mineral oil was added to each tube, which was then placed in a Perkins Elmer-Cetus thermal cycler (Norwalk, CT) and programmed for 30 cycles of 95 °C for 1 minute and 34°C for 2 minutes. At the end of the 30 cycles, the reactions were left at 72°C for 7 minutes, and then the cycler was maintained at

4°C. After confirmation of amplification by 2% agarose gel electrophoresis, the product-containing reactions were pooled, precipitated and digested with *Kpn*I and *Sac*I. Doubly restricted fragments were distinguished from single cut or uncut fragments on non-denaturing acrylamide gels, and the appropriate fragment was excised and isolated as described above.

2. Generation of Random Sequence – Containing Libraries

Cesium chloride gradient purified pMDC ("dummy" vector) which was constructed as described above in Example 1, digested with *Kpn*I and *Sac*I restriction endonucleases, and gel-isolated from a 1% agarose/lx TBE gel using GenClean II (Bio101, La Jolla, CA). This vector was ligated with the gel-isolated PCR-amplified random fragment overnight at 16°C with 1 unit of T4 DNA ligase.

3. Selection of TK Mutants

The ligated mixture was then used to transform KY895 by electroporation (BioRad gene pulser, 2kV, 25μF, 400 Ω). Briefly, cells were prepared for electroporation according to a protocol provided by BioRad (Richmond, CA). After each pulse, 1mL of SOC (2% Bactotryptone/0.5% yeast extract/10mM NaCl/2.5mM KCl/10mM $MgCl_2$/10mM $MgSO_4$/20mM glucose) was added to the curette and the electroporation mixture transferred to a 25mL snap-cap Falcon tube. After the tubes were shaken for 1 hour at 37°C, the cells were plated onto LB plates [per liter: 10g tryptone/5g of yeast extract/10g NaCl (pH 7)] containing carbenicillin (50μg/mL), ("LB+ $carb^{50}$ plates") and incubated at 37°C overnight. The number of colonies was counted, picked with a toothpick, and streaked on TK selection media [2% BBL Trypticase peptone (Becton Dickenson, Cockeysville, MD)/0.5% NaCl/0.8% Gel-Rite (Scott Laboratories, Carson, CA)/0.2% glucose/50μg/mL carbenicillin/10μg/mL 5'-fluorodeoxyuridine/2μg/mL thymidine/12.5μg/mL uridine]. The basis of this selection is that 5'-fluorodeoxyuridine (FUdR) is phosphorylated by thymidine kinase to form FdUMP, an inhibitor of the *de novo* pathway enzyme, thymidylate synthase. The requirement for dTMP can then be fulfilled only by an active thymidine kinase. Uridine is supplied to inhibit thymidine phosphorylase. After 16-24 hours, the TK selection plates were scored for growth, and any positives picked and restreaked on TK selection plates and LB + $carb^{50}$ plates to confirm the phenotype.

Approximately 260 random transformants were screened for their ability to complement KY895, a TK-deficient *E. coli* on TK selection media. Of these, 82 were scored

as positives and sequenced. Therefore, approximately 32% of all transformants encoded functional enzymes.

B. Analysis of Mutants

TK mutants were isolated and sequenced as follows. Briefly, mutant DNA was isolated from overnight cultures grown in 2 x YT (per liter: 16g tryptone/l0g of yeast extract/5g NaCl) + $carb^{50}$ using the Promega Magic miniprep kit according to the manufacturer's instructions, except that 3mLs of culture was used per isolation because of the low copy number of the plasmid. Ten microliters of each dsDNA was alkaline-denatured, precipitated, and resuspended in Sequenase reaction buffer, H_2O, and sequencing primer (5'-CATGCCTTATGCCGTGA-3') (SEQUENCE ID No. 11). The primer was then annealed, and the DNA subjected to dideoxy sequencing (Sanger et al., 1977) using Sequenase according to the manufacturers instructions (USB, Cleveland, OH).

Eleven of the clones encoded wild-type amino acid sequence (13.4%), with seven of these containing the wild-type nucleotide sequence. Three clones with wild-type amino acid residues contained single nucleotide changes (all different), and one contained three nucleotide changes. As shown in Table IA below, a total of 49 TK positive clones containing single amino acid changes (59.8%) were identified. Nineteen double amino acid mutations (23.2%), two triple (2.4%) and one clone containing four amino acid changes (1.2%) were identified. Within Table IA, wild-type HSV-1 TK amino acids mutated are given in the boldface box with the residue number and the type of residue found in the majority of sequences [O = hydrophobic; I = hydrophilic; (+) = positively charged; (-) = negatively charged residues]. Below the wild-type residue are the number of times a particular amino acid substitution was found. In the bottom section, the percentages of each type of residue found are listed.

The amino acid sequences of clones with multiple alterations are shown in Table 1B. The wild-type amino acids and their positions in the HSV-1 TK polypeptide are indicated at the top of the table. Double, triple, and quadruple amino acid substitutions are shown in the respective categories. If a set of mutations was identified more than once, the number of occurrences is noted on the left in parentheses.

TABLE IA

Wild-type Sequence	O **P** 155	O **F** 161	(-)I **D** 162	(+)I **R** 163	(+)I **H** 164	O **P** 165
Substitutions at Each Position	3L 2A 2T 1Q 1R	4I 4Y 3C 2L 1S	5E 1G	5C 1S	3N 1T	3L 2T 2S 1N 1A
Types of Substitutions	11%(+) 33% I 56% O	57% I 43% 0	83% (-)I 17% I	100% I	100% I	10% (+) 50% I 40% O

TABLE IB

Number of changes	**P** 155	**F** 161	**D** 162	**R** 163	**H** 164	**P** 165	
Doubles	A	V					(SEQ ID NO. 141)
	Q	I					(SEQ ID NO.142)
	Q		E				(SEQ ID NO. 143)
	R		E				(SEQ ID NO. 144)
(4)	R		G				(SEQ ID NO. 145)
	T		E				(SEQ ID NO. 146)
(2)		I		H			(SEQ ID NO. 147)
		I				R	(SEQ ID NO. 148)
		N				S	(SEQ ID NO. 149)
			Y	C			(SEQ ID NO. 150)
			N		K		(SEQ ID NO. 151)
(2)			E		N		(SEQ ID NO. 152)
				P	Q		(SEQ ID NO. 153)
					Q	L	(SEQ ID NO. 154)
	Q		E			L	(SEQ ID NO. 155)
Triples	A			P		T	(SEQ ID NO. 156)
Quadruple			N	S	N	A	(SEQ ID NO. 157)

C. Secondary Screening and Subcloning

The ability of pMCC (KY895) and 35 log-phase mutant pMDC (KY895) cultures to produce colonies on acyclovir ("ACV") or AZT plates was determined in a secondary screen as described below. Briefly, log-phase cultures of TK positive clones were serially diluted in 0.9% NaCl and spread onto acyclovir or AZT plates (TK selection plates except 1μg/mL thymidine + 1μg/mL acyclovir or 0.05μg/mL AZT). Mutant cultures were also spread onto duplicate TK selection and LB + carb50 plates. One set of TK selection plates and LB + carb50 plates were incubated at 42°C. All other plates were incubated at 37°C. After 16-24 hours the plates were scored.

Results are shown in Table II below. Briefly, only mutants that gave results which differed from those observed with the wild-type pMCC (KY895) are shown. Mutants are designated with the wild-type residue and position number followed by the amino acid substitution deduced from the nucleotide sequence; e.g.,

F161I (SEQ ID NO. 162) indicates that isoleucine replaces phenylalanine at residue 161 in this particular mutant. (++) indicates that the same number of colonies were observed as compared to control plates; (+) indicates that fewer (<20% those observed with pMCC) and generally smaller (~50% smaller diameter) colonies were observed as compared to control plates; and (-) indicates that no colonies were observed.

TABLE II

Clones	ACV	AZT	LB	37°C	42°C
pMCC (wild-type)	++	++	++	++	++
P155A/F161V (SEQ ID NO. 141)	++	+	++	++	++
F161I (SEQ ID NO. 162)	+	+	++	++	++
F161C (SEQ ID NO. 163)	+	-	++	++	++
F161L (SEQ ID NO. 164)	++	++	++	++	-
R163P/H164Q (SEQ ID NO. 153)	+	+	++	++	-
F1611/R163H (SEQ ID NO. 147)	++	++	++	++	+
pMDC	-	-	++	-	-

As shown in Table II, all cultures formed colonies on control TK selection and LB+carb[50] plates. In comparison to the wild-type, several mutants appeared to preferentially utilize one or both nucleoside analogues over thymidine (P155A/F161V (SEQ ID NO. 141), F161I (SEQ ID NO. 162), F161C (SEQ ID NO.163), and R163P/H164Q (SEQ ID NO.153)). In addition, several mutants were unable to form colonies on TK selection plates at 42°C (F161L (SEQ ID NO. 165) and R163P/H164Q (SEQ ID NO.153)), and one (F161I/R163H) (SEQ ID NO. 147) showed a severely reduced ability to form colonies at 42°C.

D. Expression of Mutant Enzymes in a Cell-Free Translation System

1. Subcloning of Selected Mutants

In order to study the properties of the mutant TKs, the 1.07 kbp *Mlu*I¬*Bss*HII fragment of eight mutants was subcloned into the in vitro vector pT7:HSVTKII. More specifically, DNAs of selected clones were restricted with *Mlu*I and *Bss*HII to release a 1.07kbp fragment [nucleotide numbers ~335 through 1400 on the McKnight sequence (*Nucl. Acids Res. 8:* 5949-5964, 1980; the McKnight strain was derived from the mp strain of HSV-1, Wagner, *PNAS* 78:1441-1445, 1981)]. The fragments were gel-isolated from 1% agarose gels using GenCleanII, and ligated to pT7:HSVTKII vector DNA which had been restricted with *Mlu*I and *Bss*HII, treated with calf intestinal alkaline phosphatase, and gel-isolated. pT7:HSVTKII was derived from pT7:HSVTK transcription vector described by Black and Hruby in *J. Biol. Chem. 267*:9743-9748, 1992. Briefly, pT7:HSVTKII differs from pT7:HSVTK only by the loss of an *Nco*I-*Bam*HI fragment 3' to the end of the HSV-1 tk gene which was originally used to aid in the initial cloning of the *tk* gene.

2. Sequence Analysis

In the final sequence analysis of the eight mutant fragments subcloned into the pT7:HSVTKII vector, two additional amino acid differences were identified between these tk genes. The sequence of pT7:HSVTKII is exactly the same as that published by McKnight (*Nuc. Acids Res 8*(24):5949-5963, 1980). pMCC, the parental plasmid of pMDC and hence the vector into which the random sequences were ligated, contains two amino acid aberrations from the McKnight sequence. These are at position 434 (C→T) and 575 (G→A), and result in a proline-49 to leucine and an arginine-89 to glutamine change. Therefore, all mutants contain these two mutations in addition to those described. In addition, a single nucleotide difference at position 480 (C→T) was also identified but does not result in an amino acid change.

Because all *in vitro* analyses were compared against pT7:HSVTKII as the wild-type, the *M1u*I-*Bss*H1I fragment from pMCC was subcloned into the corresponding sites of pT7:HSVTKII (now designated pT7:MCC) and the subsequent cell-free translation products compared to those derived from pT7:HSVTKII. Time course and thermal stability analyses showed no significant difference between pT7:HSVTKII-and pT7:MCC-derived translation products. No significant difference in phosphorylation efficiency was observed between pT7:MCC and pT7:HSVTKII when thymidine (1.3-fold), deoxycytidine (1.3-fold), GCV (0.8-fold), ACV (0.95-fold), or AZT (1.1-fold) were used as substrate. Furthermore, Sanderson et al. (*J. Mol. Biol. 202*:917-919, 1988) reported that the K_m for thymidine and ATP and the V_{max} of TK purified from *E. coli* harboring pHETK2 (the parent plasmid of pMCC) and HSV-1-infected cells were indistinguishable. Therefore, the alterations observed in the properties of the mutant TKs can be attributed to the nucleotide substitutions within the target region and that any differences between the vectors (pT7:MCC and pT7:HSVTKII) exerted only minor changes in catalytic properties.

3. In vitro Transcription and Translation

The transcripts described above were then used in a rabbit reticulocyte lysate cell-free translation system to synthesize active enzymes. Cell-free translation was according to Promega using nuclease-treated rabbit reticulocyte lysates.

Expression of full-length proteins was analyzed by subjecting ^{35}S-radiolabeled cell-free translation products to SDS-PAGE and autoradiography. Briefly, 1 μl of each radiolabeled cell-free translation in vitro-derived mutant mRNAs was subjected to SDS-containing polyacrylamide (12%) gel electrophoresis. An autoradiograph of this gel is shown in Figure 7. The first lane contains ^{14}C-labeled rainbow molecular weight markers (Amersham) with the apparent molecular weight (x 10^{-3}) given on the left. The second lane corresponds to a cell-free translation performed in the absence of any added mRNA. The third lane corresponds to the wild-type pT7:HSVTKII mRNA translation product. All other lanes contained translation products of the mutant mRNAs produced as described above. As is evident from Figure 7, the major radiolabeled translation product from each mutant transcript migrates during electrophoresis as a ~43kDa protein with the same electrophoretic mobility as that observed with translation products from wild-type pT7:HSVTKII transcripts.

To quantitate the level of protein synthesis for each translation, determination of trichloroacetic acid precipitable counts from each of the same samples was performed in triplicate. The amount of acid-precipitable counts roughly parallels the band intensity of each mutant in Figure 7.

E. Time Course Analysis of Mutant Enzymes

On the basis of TK activities, mutant TKs were classified into two subsets: (1) high-activity mutants (P155A/F161V (SEQ ID NO. 141), F161I (SEQ ID NO.162), F161C (SEQ ID NO. 163), and D162E (SEQ ID NO. 170)); (2) low-activity mutants (F161I/R163H (SEQ ID NO. 147), F161L (SEQ ID NO. 164), D162G (SEQ ID NO. 169), and R163P/H164Q (SEQ ID NO. 153)). For the high-activity mutant enzymes, unlabeled translation products were diluted 1/9 and incubated for 0, 5, 10, 20, or 30 minutes at 30°C. Results of this experiment are shown in Figure 8A: The TK activity results (counts per minute) were adjusted to reflect equivalent protein synthesis levels using the corresponding TCA-precipitable counts (^{35}S cpm). Two of the mutants (F161I (SEQ ID NO. 162) and P155A/F161V (SEQ ID NO. 141)) demonstrated a statistically higher affinity for thymidine than the wild-type TK. Standard deviations of F161C (SEQ ID NO. 163) and D162E (SEQ ID NO. 170) activities (data not shown) indicate no difference in activities when compared to the wild-type TK enzyme activities.

The low-activity mutants were diluted 1/5, and the rate of phosphorylation as a function of time was also determined. Results of this experiment are shown in Figure 8B. The time course analysis indicates that most of the mutants had less than 10% wild-type activity. One, F161L (SEQ ID NO. 164), however, demonstrated a moderate ability to phosphorylate thymidine, albeit at a much reduced rate from HSVTKII.

F. Thermal Stability Assays

In the assays for colony formation on TK selection plates, several mutants were unable to complement KY895 at 42°C, suggesting that these mutant TKs were temperature-sensitive. To substantiate this observation, cell-free translation products were incubated at 42°C for increasing times prior to being assayed for enzyme activity. Briefly, cell free translation ("CFT") products of each high-activity mutant, -RNA, and HSVTKII samples were diluted 1/9 and incubated for 0, 5, 10, and 20 minutes at 42°C. The preincubated samples were then assayed for 5 minutes (P155A/F161V (SEQ ID NO. 141) and F1611 (SEQ ID NO. 162)) or 20 minutes (-RNA, HSVTKII, F161C (SEQ ID NO. 163), and D162E (SEQ ID NO. 170)). The percent of activity remaining was determined with the untreated samples set at 100%. As shown in Figure 9A, except for F161C (SEQ ID NO.163), all high-activity mutants displayed thermal stabilities similar to HSVTKII after 42°C preincubation periods as long as 60 minutes (data not shown). Because F161C (SEQ ID NO. 163) lost greater than 90% of enzyme activity within the first 20 minutes at 42°C, shorter incubation periods at 42°C were performed (0, 5,

10, and 20 minutes). F161C (SEQ ID NO. 163) was exceptionally thermolabile demonstrating a ~85% activity loss after only 5 minutes at 42°C.

Low-activity mutant CFT products were diluted 1/5 and incubated for 0, 20, 40, or 60 minutes at 42°C. The preincubated samples were then assayed in triplicate for the thymidine phosphorylation for 60 minutes. The percent of activity remaining was determined using the untreated (time 0) sample as 100%. As shown in Figure 9B, for the low-activity mutant subset one translation product (F161L (SEQ ID NO. 164)) was more thermolabile that HSVTKII. Others in this set (R163P (SEQ ID NO. 171), F161I/R163H (SEQ ID NO. 147), H164Q (SEQ ID NO. 172), and D162G (SEQ ID NO. 169)) were equivalent to HSVTKII.

G. Substrate Specificity Assays

Three of the mutants (P155A/F161V (SEQ ID NO. 141), F161I (SEQ ID NO. 162) and F161C (SEQ ID NO. 163)) were assayed in triplicate for the relative levels of phosphorylation using thymidine, deoxycytidine, ACV, GCV, or AZT as substrates. Briefly, forty-eight micromoles of each tritiated substrate was used in each assay reaction. Translation products were diluted for each nucleoside assay as follows (translation/H_2O): 1/100, thymidine; 2/3, deoxycytidine, GCV, and AZT; 4/1, ACV. Each set of assays was incubated for 2 hours at 30°C and the amount of phosphorylated product determined.

The counts per minute of each set of assays were adjusted, and plotted as 30 shown in Figure 10. Briefly, both P155A/F161V (SEQ ID NO. 141) and F161I (SEQ ID NO. 162) displayed an elevated capacity to phosphorylate thymidine relative to HSVTKII, 2.6- and 2.2-fold, respectively. Phosphorylation of deoxycytidine by the mutant enzymes ranged from 1.9- to 2.8-fold over the wild-type enzyme (F161I (SEQ ID NO.162), 1.9-fold; F161C (SEQ ID NO. 163), 2.8-fold; P155A/F161V (SEQ ID NO. 141), 2.8-fold). Two mutants appeared to share an increased ability to phosphorylate ACV (2.4- and 2-fold over HSVTKII by F155A/F161V (SEQ ID NO. 141) and F161C (SEQ ID NO.163), respectively). All mutants demonstrated approximately wild-type levels of AZT phosphorylation. All mutants assayed appeared to share a large increase in GCV phosphorylation at 3.9-5.2-fold compared to wild-type phosphorylation levels.

EXAMPLE 4

ANALYSIS OF TK MUTANTS WITH ALTERED CATALYTIC EFFICIENCIES

In order to identify mutants with altered catalytic activity, 190 of the TK mutants isolated in Example 1 (TKF) were analyzed in the assays set forth below.

A. Colony Formation Ability As A Functional Thymidine Uptake

The protein content of the purified enzymes was estimated by a modification of the Bio-Rad protein assay. A standard curve was established using BSA and 25 μl of Bio-Rad reagent in a final volume of 125u1. The amount of protein was determined by measuring the OD at 595 nm and comparing it to that of BSA.

In order to identify mutants with altered TK activity, a secondary screening protocol was designed based on the ability of the mutants to grow on medium containing different concentrations of thymidine (Table I). Briefly, it was first established that 1.0 and 10.0 μg/mL are the minimum and maximum concentrations of thymidine in the medium that supports the growth of *E. coli* harboring the wild-type *tk* plasmid. Since *E. coli* harboring the wild-type plasmid are unable to form visible colonies on TK-selection medium containing low thymidine (0.05 μg/mL), it was postulated that growth at this thymidine concentration might be indicative of mutants with an increased ability to phosphorylate thymidine. Accordingly, 0.05 μg/mL thymidine was used to select for variants with high TK activity and 20 μg/ml thymidine for variants with low activity.

Table I below shows the ability of selected mutants to functionally complement *tk⁻ E. coli* KY 895 as a function of increasing thymidine concentration. When all the 190 TK variants and the wild-type were subjected to screening at the thymidine concentrations indicated in Table I, only one, TKF 36 (SEQ ID NOs. 14 and 15), formed colonies at the lowest thymidine concentration tested (0.05 μg/mL). On the other hand, only TKF 41 (SEQ ID NOs. 16 and 17) grew at the highest concentration of thymidine in the medium. All of the other 188 mutants and the wild-type formed visible colonies on medium containing 1 μg/mL thymidine.

TABLE I

COLONY FORMING ABILITY OF *TK⁻ E. COLI* KY895 TRANSFORMED WITH WILD-TYPE AND MUTANT PLASMIDS, AS A FUNCTION OF THYMIDINE CONCENTRATION

Mutant	Thymidine concentration (μ/mL)[a]				
	0.05	1	2	10	20
Wild-type	_a	+a	+	±[b]	-
TKF 36	+	+	+	±	-
TKF 41	-	-	+	+	+[c]
		+			

TKF 52	-	+	+	+	-
TKF 99	-	+	+	+	-
TKi 208[d]	-	+	+	+	-

Colony formation was determined after incubation at 37°C for 24 hours.

[a] + and - indicate the ability or inability of *E. coli* harboring different plasmids to form visible colonies on the indicated TK-selection media.

[b] ± indicates initial cell growth: cell death was apparent after incubation for 20 hours and may be due to the nucleotide pool imbalance generated by excessive phosphorylation of thymidine in the mutant and wild-type clones.

[c] Since TKF 41 seemed to be a very low activity clone, overexpression of this mutant TK was necessary for the survival of *E. coli* on TIC-selection medium. pMCC and pMDC expression vectors have a temperature-sensitive repressor gene c1857 which becomes inactive at 42°C and, hence, there is overexpression of TIC and subsequent cell death. In order to obtain controlled expression, screening was performed at 37°C. However, TKF 41 containing E. coil was incubated at 42°C on 20 μg/mL thymidine containing TK-selection medium.

[d] TKI 208 was obtained from the library described above in Example 2.

B. Sequence Analysis of High and Low Activity Clone

Wild-type *tk* and selected mutants were sequenced as described above in Example 2. Table II shows the nucleotide and deduced amino acid sequences of the wild-type *tk* and selected mutants for codons 165 to 175. Briefly, TKF 36 (SEQ ID NOs. 14 and 15), the mutant that forms colonies on low thymidine-containing medium, contains only a single amino acid substitution (Ala168→Ser), whereas TKF 41 (SEQ ID NOs. 16 and 17) contained four substitutions: Pro165 →Ser, Ala167→Gly, Leu170→Gln and Ala174→Val. Interestingly, TKF 52 (SEQ ID NOs. 18 and 19) has a different amino acid substitution (Ala168→Thr) at the same position as TKF 36 (SEQ ID NOs. 14 and 15), but is unable to form colonies on low thymidine-containing medium. TKF 99 (SEQ ID NOs. 20 and 21) contains two amino acid substitutions (Cys 171→Leu and Ala 174→Thi). TKI 208 has a single nucleotide substitution which results in a Leu170→Val substitution.

TABLE II

NUCLEOTIDE AND DEDUCED AMINO ACID SEQUENCES OF THE WILD-TYPE AND MUTANT TK ENZYMES AT THE TARGET REGION

	165a	166	167	168	169	170	171	172	173	174	175	SEQ ID
Wild-type	ccc	atc	gcc	gcc	ctc	ctg	tgc	tac	ccg	Gcc	gcg	12
	pro	Ile	Ala	Ala	Leu	Leu	Cys	Tyr	Pro	Ala	Ala	13
TKF36	ccc	Atc	gcc	Tcc	ctc	ctg	tgc	tac	ccg	Gcc	gcg	14
	Pro	Ile	Ala	SER	Leu	Leu	Cys	Tyr	Pro	Ala	Ala	15
TKF41	Tcc	atc	gGc	gcc	ctA[b]	cAG	tgc	tac	ccg	gTc	gcg	16

	SER	Ile	GLY	Ala	Leu	GLN	Cys	Tyr	Pro	VAL	Ala	17
TKF52	ccc	atc	gcc	**Acc**	ctg	ctg	tgc	tac	ccg	gcc	gcg	18
	Pro	Ile	Ala	**THR**	Leu	Leu	Cys	Tyr	Pro	Ala	Ala	19
TKF99	ccc	atc	gcc	gcc	TtA	ctg	tTA	tac	ccg	Acc	gcg	20
	Pro	Ile	Ala	Ala	Leu	Leu	LEU	Tyr	Pro	THR	Ala	21
TKI208	ccc	atc	gcc	gcc	ctc	Gtg	tgc	tac	ccg	gcc	gcg	22
	Pro	Ile	Ala	Ala	Leu	VAL	Cys	Tyr	Pro	Ala	Ala	23

[a]Shows the codon number of the target region that was degenerated. The wild-type nucleotide and amino acid sequences are shown below the codon number.

[b]The silent mutations. No other nucleotide changes were observed in the region sequenced (spanning codons 140-182). Each template was sequenced twice.

Substituted nucleotide and amino acid residues are shown in bold capital letters.

C. Tymidine Uptake in *E. coli* HarboringWild-tvpe and Mutant TK Plasmids

In order to ascertain the actual level of thymidine uptake in *E. coli* harboring wild-type or mutant plasmids, the following assays were performed.

1. [Methyl-^{3}H]thymidine uptake assay

[Methyl-^{3}H]thymidine uptake in *E. coli* harboring wild-type or mutant plasmids was determined essentially as follows. Briefly, overnight cultures of *E. coli* containing pMDC (inactive TK), a plasmid containing wild-type TK, or TK36 were diluted 1:100 with LB-medium containing 100 μg/mL of carbenicillin, grown to 0.1 OD at A_{550}, shifted to 37°C and incubated with vigorous shaking. Once an OD of 1.0 was attained, the culture was brought to room temperature (~25°C) and thymidine was added to 1.0 mL aliquots at a final concentration of 0.21 μM (0.16 μCi [*methyl* ^{3}H]thymidine). After incubation for 0, 5, 10,. 20, 30 and 60 s at 22°C, 50 μl aliquots were transferred onto nitrocellulose filters (0.45 μm), washed under vacuum with 10 mL of chilled 50 mM Tris-HC1, pH 7.4, 0.9% NaCl, dried and counted in a scintillation counter using scintiverse BD (Fisher). Results are shown in Figure 11. Briefly, there was essentially no thymidine uptake in *E. coli* harboring pMDC. The amount of hymidine uptake in *E. coli* harboring TKF 36 (SEQ ID NOs. 14 and 15) was 42% greater than in *E. coli* harboring the wild-type plasmid (18 pmol/10^8 cells compared to 12.7 pmol/10^8 after incubation for 10 s).

2. Incorporation of [methyl-^{3}H]thymidine into acid-insoluble material

The amount of TK activity in crude *E. coli* extracts containing the wild-type and mutant plasmids was determined indirectly by measuring the incorporation of thymidine into acid-insoluble material.

Briefly, cultures were grown as described above under section 1. To 0.5 mL of culture, thymidine was added to a final concentration of 1.32 μM (0.2 μCi [*methyl*-^{3}H]thymidine). A 30 μl aliquot was taken out after designated times of incubation and added to 2.0 mL of cold 5% perchloric acid. The precipitate was washed and radioactivity incorporated into an acid-insoluble material was determined essentially as described by Dube et al., 1991.

Figure 12, shows that the incorporation of [*methyl*-^{3}H]thymidine into an acid-insoluble product is more rapid with TKF 36 (SEQ ID NOs. 14 and 15) *E. coli* than with *E. coli* harboring the wild-type plasmid or the other *tk* mutants tested. One of the mutants, TKF 99 (SEQ ID NOs. 20 and 21), having two amino acid substitutions (Cys171→Leu and A1a174→Thr) exhibited the same rate of thymidine incorporation as did the wild-type. TKF 52 (SEQ ID NOs. 18 and 19) contains an A1a168→Thr substitution (compare A1a168→Ser in TKF 36 (SEQ ID NOs. 14 and 15)) and is unable to form colonies in the lowest thymidine-containing TK-selection medium (Table I), yet incorporates thymidine into acid-insoluble material at a rate greater than that of wild-type but less than that of TKF 36 (SEQ ID NOs. 14 and 15).

D. Purification of Wild-types and Mutant TKS

Crude extracts of the different mutants were obtained from 11 cultures that were grown at 30°C to 0.1 OD at A_{550}, shifted to 37°C and grown to 1.0 OD. The cells were harvested by centrifugation at 4°C, washed with 25 mL of a solution containing 25% (w/v) sucrose, 50 mM Tris-HC1, pH 7.5, and 5 mM EDTA. After centrifugation the cell pellet (~5-6 g weight) was stored at -70°C. The cell pellet was thawed and suspended in 20 mL of buffer I (buffer I consisted of 10 vol. 50 mM Tris-HC1, pH 7.5, 10% sucrose mixed with 1 vol. 0.3M spermidine-HC1, 2.0M NaCl, 10% sucrose and 0.5 mM PMSF, pH 7.5). Once resuspension was uniform, 4.0 mL of buffer I containing 6.25 mg of lysozyme was added. The suspension was poured into a chilled centrifuge tube and placed on ice for 30 minutes. If cells did not lyse within 30 minutes, the tube was placed in a 37°C waterbath for 4-6 minutes to enhance lysis. Once cells started to lyse as judged by increasing stringiness, 2-3 mL of chilled buffer I containing 50 μg/mL aprotinin and 2 μg/mL of each leupeptin and pepstatin, was added to a final volume of 25 mL and the mixture was centrifuged at 28,000 r.p.m. for 1 hour at 4°C and the supernatant was stored at 70°C.

The wild-type and mutant TKs were purified by affinity chromatography on a matrix of *p*-aminophenylthymidine 3'-phosphate coupled to CH-Sepharose 4B (Pharmacia) as described by Kowal and Marcus (*Prep. Biochem. 6*:369-385, 1976) with modification by Lee and Cheng (*J. Biol. Chem. 251*:2600-2604, 1976). All buffers used in the purification of TK contained 5mM DTT, 50 μ/mL aprotinin, 2 μg/mL each of leupeptin and pepstatin and 1 mM PMSF unless otherwise indicated. A 7 mL bed-volume column was equilibrated with buffer A (0.1 M Tris-HCl, pH 7.5, 10% glycerol) and then loaded with ~25 mL of the unfractionated supernatant at a rate of 8-10 mL/h. The column was recirculated with the flow-through twice and then washed sequentially with ten bed-volumes each of buffer B (0.1 M Tris-HCl, pH 7.5, 0.5 M KCl, 10% glycerol) followed by buffer A. TK was eluted with a linear gradient of thymidine (0-600 μM) using 30 mL each of buffer A and buffer C (0.3 M Tris - HCl, pH 7, 4, 50 mM KCI, 10% glycerol). TK assay was performed on all the fractions and peak TK fractions were pooled and dialyzed against three changes of 21 of dialysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM DTT, 10% glycerol). In the final dialysis, protease inhibitors were omitted from the buffer and the dialyzed fractions were aliquoted and stored at -70°C. The column was washed thoroughly twice by using the same washing and elution protocols as described above prior to application of each extract preparation.

The protein content of the purified enzymes was estimated by a modification of the Bio-Rad protein assay. A standard curve was established using BSA and 25 μl of Bio-Rad reagent in a final volume of 125ul. The amount of protein was determined by measuring the OD at 595 nm and comparing it to that of BSA.

[Methyl-3H]thymidine uptake

Results are shown in Figure 11. Briefly, there was essentially no thymidine uptake in *E. coli* harboring pMDC. The amount of thymidine uptake in *E. coli* harboring TKF 36 (SEQ ID NOs. 14 and 15) was 42% greater than in *E. coli* harboring the wild-type plasmid (18 pmol/10^8 cells compared to 12.7 pmol/10^8 after incubation for 10 s).

The amount of TK activity in crude E. coli extracts containing the wild¬type and mutant plasmids was determined indirectly by measuring the incorporation of thymidine into acid-insoluble material.

E. Kinetic Parameters Of Purified Mutant Thymidine Kinases

The three cellular parameters so far studied suggest that TKF 36 (SEQ ID NOs. 14 and 15) is a more active enzyme than any of the other mutant enzymes tested or the wild-type. In order to determine the kinetic parameters of catalysis, wild-type, TKF 36 (SEQ ID NOs. 14 and 15) and three other mutant thymidine kinases were purified to near homogeneity

using affinity chromatography as described above. The purified wild-type, TKF 36 (SEQ ID NOs. 14 and 15) and TKI 208 (SEQ ID NOS. 22 and 23) were examined by electrophoresis in an SDS-PAGE system and were found to exhibit a single prominent band that migrated at 43 kDa, which was judged to be 95% homogeneous by silver staining.

Kinetic parameters were determined essentially as described below. Briefly, TK assay mixtures (50 μl) contained 50 mM Tris-HC1, pH 7.5, 5 mM ATP, 4 mM $MgC1_2$, 2.5 mM DTT. 12 mM KC1, 018 mg/mL BSA, 5% glycerol, 1 μM thymidine (0.3 μCi [*methyl*-^{3}H]thymidine) and the indicated amounts of purified enzymes. The kinetics of thymidine phosphorylation were determined by varying the unlabeled thymidine concentration (0-4.0 μM) and known amount of purified enzymes (the sp. acts of the purified TKs were 1.1, 3.0, 0.5, 0.34 and 0.01 units for wild-type. TKF 36 (SEQ ID NOs. 14 and 15), TKI 208 (SEQ ID NOS. 22 and 23), TKF99 (SEQ ID NOs. 20 and 21) and TKF41 (SEQ ID NO. 16 and 17), respectively). One unit of enzyme is defined as the amount that phosphorylates 1.0 pmol of thymidine to thymidylic acid in 1 minute under the conditions described above. Incubation was at 34 ± 1°C for 10 minutes. The reaction was stopped by the addition of 1 mM cold thymidine. Half of the reaction mix was pipetted onto a DEAE-cellulose disc (25 mm) and the disc was dipped in distilled water (1 minute) followed by four washes each in 10 mL of absolute ethanol. The adsorbed products on the disc were counted in a scintillation counter. The kinetic parameters K_m and V_{max} were determined by using the Cleland SUBIN program (Cleland, *Methods Enzymol. 63*:103-138, 1979) and the values for k_{cat} were calculated from the equation $V_{max} = k_{cat}$[E]o, where [E]o is the total enzyme concentration.

Results of these assays are summarized in Table III. A1a168→Ser substitution in TKF 36 (SEQ ID NOs. 14 and 15) resulted in a 4.8-fold enhancement in k_{cat}. None of the other purified mutant enzymes (TKF 41 (SEQ ID NO. 16 and 17), TKF 99 (SEQ ID NOs. 20 and 21) and TKI 208 (SEQ ID NOS. 22 and 23)) that were analyzed exhibited an increase in k_{cat} compared to that of the wild-type TK. A 2.2-fold decrease in k_{cat} results form the Leu170→Val substitution in TKI 208 (SEQ ID NOS. 22 and 23), whereas two of the other *tk* mutants, TKF 99 (SEQ ID NOs. 20 and 21) and TKF 41 (SEQ ID NOs. 16 and 17), with decreased efficiencies in the *in vivo* assays, exhibited a 28- and 34 700-fold decrease in k_{cat}. Table III also presents the Michaelis constant (K_m) for the mutants and wild-type with thymidine as a substrate. The apparent K_m for the wild-type enzyme was 0.47 μM, which agrees well with previously reported values (Jamieson and Subak-Sharpe, *J. Gen. Virol. 24*:481-492, 1974; Elion, *Am. J. Med. 73*:7-13, 1982; Waldman et al., *J Biol. Chem. 258*:11571-11575, 1983). Even though TKF 36 (SEQ ID NOs. 14 and 15) showed a higher

k_{cat} value its affinity for thymidine, as reflected in the K_m, is 6.2-fold lower than the wild-type TK. TKI 208 (SEQ ID NOS. 22 and 23), TKF 41 (SEQ ID NOs. 16 and 17) and TKF 99 (SEQ ID NOs. 20 and 21) have a similar K_m to that of the wild-type. Interestingly, the k_{cat}/K_m value of TKF 36 (SEQ ID NOs. 14 and 15) [2.0×10^6 $s^{-1}M^{-1}$] is not very different from the wild-type [2.5×10^6 $s^{-1}M^{-1}$] , while TKI 208 (SEQ ID NOS. 22 and 23), TKF 99 (SEQ ID NOs. 20 and 21) and TKF 41 (SEQ ID NOs. 16 and 17) exhibit lower values of 1.57×10^6, 0.15×10^6 and 0.00012×10^6 $s^{-1}M^{-1}$, respectively.

TABLE III

COMPARISON OF KINETIC PARAMETERS OF THE THYMIDINE KINASES

Enzyme	K_m (μM)	k_{cat} ($^{1/s}$)
Wild-type	0.47 ± 0.1[a]	1.2
TKF 36	2.90 ± 0.01	5.7[b]
TKF 41	0.28 ± 0.16	3.5×10^{-5}[b]
TKF 99	0.29 ± 0.002	0.04[b]
TM 208	0.35 ± 0.008	0.5[b]

[a]Data presented as ± SE.

[b]The P value is <0.02 compared to the wild-type.

EXAMPLE 5

SELECTIVE KILLING OF CELLS TRANSFECTED WITH RETROVIRAL VECTORS CONTAINING MUTANT HSV-1 TK

The example describes the construction of retroviral vectors which express a type 1 Herpes Simplex Virus thymidine kinase, a proline to alanine mutation at position 155, and a phenylalanine to valine mutation at position 161.

A. Vector Construction

The thymidine kinase gene from P155A/F161V (SEQ ID NO. 141) is utilized to replace the wild-type HSV tk sequences in the Moloney Murine Leukemia Virus ("MoMLV") based vector G1TkSvNa.90 from Genetic Therapy, Inc. (Gaithersburg, MD; see Ram et al. *Cancer Research 53*:83, 1993). In particular, the mutant *tk* gene is inserted downstream from the 5' long terminal repeat sequence, which the *tk* gene uses as a promoter.

This vector also contains an neomycin phosphotransferase gene (neo) which is expressed from an SV40 early promoter.

B. Producer Cell Line

The retroviral vectors described above may then be packaged by the amphotropic retroviral packaging cell line GP+envAml2 (U.S. Patent No. 5,278,056) after calcium phosphate transfection. A vector containing the gene for ß-galactosidase is used as a control vector. The cloned vector producer cells are maintained in culture containing Dulbecco's modified Eagle's medium with 10% fetal calf serum, 2mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin and 2.5 μg/ml Fungizone. Prior to administration, the media is removed and the cells rinsed with saline. The monolayers are trypsinized for 5-10 minutes at 37°C, collected, washed twice and resuspended at 5-10 x 10^8 cells/ml.

C. *In Vitro* Sensitivity to GanciclQyit

To assess the sensitivity of cells transduced with the mutant or the wild¬type tk gene containing vectors, rat 9L glioma cells and human U251 glioblastoma cells are transduced *in vitro* by exposing the cells to supematant containing replication incompetent vector particles. The transduced cells are selected by including G148 (1 mg/ml) in the culture medium. Nontransduced, HSV *tk* wild-type transduced and HSV *tk* mutant transduced cells are then evaluated for their sensitivity to increasing levels of ganciclovir. The level of DNA synthesis is determined by tritiated thymidine incorporation after various ganciclovir exposure times and ganciclovir levels. Cell viability is determined by plating the cells in 10 cm tissue culture plates in the absence or presence of various ganciclovir concentrations, and counting the number of cells at 24 hour intervals.

D. *In Vivo* Transduction

The efficiency of *in situ* transduction of and relative level of vector gene expression in the tumor cells is determined using the ß-galactosidase containing vector. Briefly, Fischer 344 rates are anesthetized and injected with 4 x 10^4 syngeneic 9L gliosarcoma cells using a 10 μl Hamilton syringe connected to a stereotaxic injection apparatus. After ten days, the same stereotaxic position is used to directly inject 1.5 x 10^6, 3 x 10^6 or 6 x 10^6 HSVtk (wild-type or mutant) ß-galactosidase transduced or nontransduced producer line cells, and producer cell line supernatants into the 9L tumor. As a control, rats are injected with the same volume of sterile saline instead of cells. Ganciclovir is then administered and the rats are sacrificed to determine the anti-tumor effect. A histological examination is also performed.

E. Dose Optimization of Ganciclovir

Rats are injected intracerebrally with 4 x 10^4 HSVtk (wild-type or mutant) or (ß-galactosidase transduced rat 9L producer cells. Seven days post inoculation, ganciclovir is administered i.p. at 5, 20 or 15 mg/kg twice daily for 7 days. Control rats receive i.p. saline injections. All rats are sacrificed after the ganciclovir treatment and the brains and tumors removed for weight determination and histological examination.

F. Tumor Regression with Wild-type and Mutant HSV tk Transduction and GCV

Based on the results of the ganciclovir dose optimization, rat tumors inoculated with transduced or nontransduced producer cells or produced cell supernatant are administered ganciclovir doses for a specific time period. Antitumor effects are determined by determination of tumor weight and histological examination.

EXAMPLE 6

THE USE OF VZV TK MUTANTS AS TARGETS FOR SELECTABLE HOMOLOGOUS RECOMBINATION

This example describes the use of a mutant Varicella Zoster Virus thymidine kinase ("VZV tk") as a target for homologous recombination in the construction of stable transfected cells lines, strains or recombinant viruses. In particular, the construction of vaccinia viruses as cloning vectors containing mutant VZV TKs for the selection of recombinant viruses in TK^+ cell lines is described.

A. Construction of Recombinant Vaccinia Virus Plasmids Containing VZV TK Mutants

VZV tk genes (wild-type and mutant) are cloned into a recombinant plasmid behind the vaccinia virus 7.5 K promoter for constitutive gene expression. In addition the neomycin phosphotransferase gene is cloned after the 3' end of the VZV *tk* gene to serve as a selectable marker. The 5' or 3' regions of the vaccinia virus encoded thymidine kinase gene flanks the 5' end of VZV *tk* gene and the 3' end of the neomycin phosphotransferase gene (neo). This allows for the insertion of the VZV tk gene into the viral genome and the concomitant inactivation of the vaccinia thymidine kinase gene. The remainder of the plasmid is based on pUC and contains an ampicillin resistance gene and a ColE1 origin of replication for maintenance of the plasmid in *E. coli*.

B. Construction of Recombinant Poxviruses

The VZV tk (wild-type or mutant) + neo recombinant plasmid or recombinant plasmid containing only the neo gene is cotransfected with the wild-type vaccinia virus into BSC40 cells. Recombinant viruses are selected by resistance to G418. After several rounds of plaque purification, the recombinant viruses are subjected to plaque hybridization and DNA analysis in order to confirm the insertion and location of the foreign genes.

C. Dose Optimization of Ganciclovir

Vaccinia virus infected and uninfected BSC40 cells are subjected to treatment with various doses of ganciclovir in order to determine the tolerance level. Cells infected with recombinant viruses expressing VZV TKs and neo or those expressing only neo will be grown in the presence of various levels of ganciclovir. VZV tk gene containing viruses are more sensitive to ganciclovir treatment than the cells alone or those infected with wild-type vaccinia virus. A level of ganciclovir is selected from the results of this experiment to select for the loss of sensitivity to ganciclovir for homologous recombination with other genes to be inserted into the VZV tk locus.

D. Selection of Recombinant VZV tk Poxviruses Using Ganciclovir

BSC40 is infected with the VZV tk recombinant virus in the presence of a recombinant plasmid carrying the gene to be introduced into the VV genome, abutted to the VV 7.5 K promoter cloned with VZV tk sequences flanking. Recombinant virus is selected with ganciclovir.

Any cell line stably transfected with the VZV tk gene can be the target for introduction of foreign genes by homologous recombination and for the selection of such an event by resistance to ganciclovir.

EXAMPLE 7

CONSTRUCTION AND ANALYSIS OF HSV-1 THYMIDINE KINASE AND HSV-1 DNA POLYMERASE VECTORS

A. Construction of Vectors

Three constructs were made containing either the HSV-1 DNA polymerase gene, HSV-1 thymidine kinase gene or both.

a) pHSG576:HSVpol

The 5.5 kb *Hin*DIII/*Eco*RI fragment from pGEM2-702 (David Dorsky, Univ. of Conn.) was cloned into pHSG576 (Sweasy and Loeb, *J. Biol. Chem 267*:1407¬1410, 1992) in two steps:

1) The 2.4 kb *Pst*I/*Eco*RI fragment was cloned into pHSG576igested with Pstl and EcoRI. This clone was designated pHSG576: 1/2 poi.

2) The 3.1kb HinDHI/Pstl fragment of HSV DNA polymerase was cloned into pHSG576:1/2 pol digested with *Hin*DIII and *Pst*I. This clone was designated pHSg576:HSV DNA pol.

b) pHSG576:HSV-1 TK

The *Xba*I/*Bam*III fragment from pET23d:HSVTK (contains the HSV-1 TK Ncol-Ncol fragment in pET23d, Novagen) was blunt-ended and cloned into the *Sma*I site of pHSG576. The clone was designated pHSG576:HSV-1TK.

c) pHSG576:HSV pol/TK

This clone contains both the HSV-1 DNA polymerase and TK genes for coexpression from the same vector. It was created in a two step cloning protocol.

1) The *Xba*I/*Bam*HI - bluntended TK fragment was cloned into the bluntended *Eco*RI site of pHSG576:1/2po1 (contains the 2.4kb *Pst*I/*Eco*RI fragment).

2) The 3.1kb *Hin*DIII/*Pst*I fragment (5' end of the polymerase gene) was cloned into pHSG576:1/2pol/TK digested with *Hin*DIII and *Pst*I. This clone was designated pHSG576:HSVpo1/TK.

B. Transformation of *E. coli* With A DNA Polymerase Defect

E. coli JS200 (polAl2recA718) was transformed with pHSG576:HSV DNA pol or pHSG576 DNA and plated on nutrient agar (NA) containing tetracycline (12.5 μg/mL) and chloramphenicol (34μg/mL). Plates were incubated at 30°C (permissive temperature). Single colonies were grown overnight in NB + tet + Cm. DNA was isolated from these cultures and used to transform JS200 again. From the second transformation several colonies from each were picked and used to inoculate NB 30 + tet + Cm in the presence or absence of IPTG. After overnight growth at 30°C, a single loopful of each culture was spread in a diverging spiral of increasing dilution from the center of the plate. NA plates + tet + Cm +/- IPTG were incubated at 30°C (permissive) or 37°C (nonpermissive).

The growth pattern of cells containing pHSG576:HSV DNA pol displayed growth of single colonies (low cell density) at 37°C, while cells containing only the vector were unable to grow at low cell density at the nonpermissive temperature.

These results demonstrate that the Herpes DNA polymerase can complement the *E. coli* Poll defect *in vivo*.

EXAMPLE 8

CONSTRUCTION AND ANALYSIS OF TK MUTANTS WITH MUTATIONS AT CODONS 159 TO 161 AND 168 TO 170 UTILIZING A 100% RANDOM LIBRARY

This example describes the construction and analysis of TK mutants that are mutagenized at codons 159 through 161 and 168 through 170.

Bacterial Strains. SY211 (BL21(DE3) tdk$^-$, pLysS) is cured of pLysS by repeated passages on non-selective plates (no chloramphenicol). (SY211 is a gift from William Summers, Yale University, New Haven, CT and is described in Summers, W. C. and Raskin, P., *J. Bact. 175*:6049-6051, 1993). The resulting strain BL21(DE3) *tdk* is used in the genetic complementation assays for thymidine kinase activity. Other strains used are described in Example 3.

Cells. BHK tk- (ts13) cells (ATCC No. CRL-1632) are purchased from the American Type Culture Collection and cultured in DMEM + 10% calf serum at 37°C under 6% CO_2.

Materials. As described in Example 3.

A. Generation of TK Mutants

1. Construction of Random Insert

Two oligonucleotides are synthesized by Operon (Alameda, CA) : MB126 (58mer) 5'-TGGGAGCTCA CATGCCCCGC CCCCGGCCCT CACCNNNNNN NNNGACCGCC ATCCCATC-3' (SEQUENCE ID No. 24) and MB127 (51mer) 5'-ATAAGGTACC GCGCGGCCGG GTAGCANNNN NNNNNGGCGA TGGGATGGCG G-3' (SEQUENCE ID No. 25). The N designates an equimolar mix of all four nucleotides during synthesis.

The purification of oligonucleotides, annealing, extension and 10 amplification by PCR is essentially as described in Example 3.

2. Generation of Random-Sequence Containing Libraries Vector Construction

pET23d, purchased from Novagen, is the backbone for the construction of pET23d:HSVTK-Dummy. pET23d:HSVTK-Dummy is used in place of pMDC (described in Example 1 and 3) for insertion of random sequences. Briefly, a 1.7kb *NcoI/Hin*DIII fragment is purified from a restriction digest of pT7:HSVTKII (Example 3) and cloned into pET23d restricted with the same enzymes to generate pET23d:HSVTK. The dummy vector is constructed by replacing the tk sequences between the *Kpn*I and *Sac*I sites with the *KpnI/Sac*I fragment from pMDC (Example 3).

Library Construction

Qiagen column purified pET23d:HSVTK-Dummy DNA is restricted with *Kpn*I and *Sac*I and the vector gel isolated using GenCleanII (Bio101, La Jolla, CA) to remove the small insert fragment. This vector is ligated with the gel isolated PCR-amplified random fragment overnight at 16°C with T4 DNA ligase.

3. Selection of TK Mutants

The ligated mixture is then used to transform BL21(DE3) *tdk* cells by electroporation as described in Example 3. The transformants are plated directly onto TK selection plates (Example 3) with a small fraction plated on 2 x YT (16g tryptone/l0g yeast extract/5g NaCl/15g BactoAgar per liter) + carbenicillin at 50μg/ml (carb^{50}) to determine the total number of transformants. The plates are incubated at 37°C overnight and scored for growth on TK selection plates and the transformation frequency determined. Colonies that grew on the TK selection plates are picked and restreaked on fresh TK selection plates and 2 X YT + carb^{50} plates. Approximately 426 positive clones are identified from a library of 1.1 x 10^6 transformants or 0.039% of all transformant conferred TK activity to *E. coli* BL21(DE3) *tdk* (Figure 14).

B. Analysis of Mutants

1. Sequence of Selected and Unselected Clones

Seventeen clones that demonstrated TK activity (selected) or are taken from 2 x YT + carb^{50} plates (unselected) are successfully sequenced. DNA is isolated using Qiagen miniprep kits and subjected to double strand sequencing as described in Example 3. Figure 15 shows the sequences from each group and demonstrates that the initial random oligonucleotides are randomized. In both selected and unselected tk genes, the introduction of secondary mutations at sites distal to the randomized region are observed. However, the mutations are primarily confined to two codons, 155 and 156. These mutations are most likely introduced by contamination during the synthesis of the original random oligonucleotides. All

changes at codon 155 are silent. Changes at codon 156 resulted in alanine to valine, serine or proline alterations. Alignment studies indicate that position 156 is not conserved either for alanine nor for the type of amino acid at that position. Therefore, it is unlikely that these secondary mutations result in any real effect on the enzyme activity of the mutants. All selected mutants contained at least two amino acid changes.

2. Secondary Screening for GCV and ACV Sensitivity

Each of the 426 mutants is picked and used to inoculate 200μl of TK selection medium (Example 3) in a 96 well microtiter plate format. All 426 clones are then serially diluted 10^4 in 0.9% NaCl with a 48-prong replicator (Sigma, St. Louis, MO). 30μl of the last dilution is spread onto TK selection plates containing 1μg/ml thymidine plus varying concentrations of ganciclovir or acyclovir. Initially 2μg/ml GCV is used and the clones unable to grow are scored as positives since any mutant with increased conversion of a pro-drug to an active toxin results in lethality. On 2μg/ml GCV 197 clones are identified. Sequential plating on 1μg/ml and 0.5μg/ml GCV lead to the identification of 47 mutants. Plating on ACV plates (1μg/ml) gave 116 ACV sensitive clones. To ensure that the clones are truly sensitive to the nucleoside analog and not simply scored because of the inability to grow on the lower thymidine concentrations used, the 47 GCV and 116 ACV clones are plated on TK selection plates containing thymidine at 1μg/ml (no nucleoside analog). Almost half of the clones are unable to grow on low thymidine for a total of 26 GCV sensitive mutants and 54 ACV sensitive mutants. Results are shown in Figure 16.

C. *In Vitro* Analysis

1. In Vitro Transcription and Translation.

Plasmid DNA is purified by Qiagen column chromatography. Transcription and translation of the 80 selected mutants is done as in Example 3 except that the isolated plasmids are not linearized prior to transcription. *In vitro* translation products are assayed in duplicate for thymidine, ganciclovir and acyclovir phosphorylation and compared to pET23d:HSVTK mRNA translation product assays (see Example 3).

2. Measurement of Enzyme Activity

Radiolabelled nucleosides are present in each assay at 1μM, 7.5μM and 7.5μM for thymidine, ganciclovir and acyclovir, respectively. The level of activity is adjusted to reflect the level of protein synthesis as determined from the TCA precipitable counts from a

duplicated translation with ^{35}S methionine. For the majority of the 80 mutant enzymes, the level of thymidine, ganciclovir and acyclovir is less that 1% that of the wild-type TK. Ten mutant enzymes displayed greater that 10% phosphorylation with at least one of the nucleosides assayed. The nucleotide sequences are shown in Figure 17. Several of the clones contained mutations outside the randomized region. Two clones, 30 and 84, have mutations that result in amino acid changes, A152V (SEQ ID NO. 122) and A156S (SEQ ID NO. 125), respectively. Four clones contain in-frame deletions; three (226, 340 and 411) with -3 deletions and one (197) with a -6 deletion. All these mutations are centered around a GC-rich region which encodes for the peptide A P P P A. This proline rich peptide is likely to comprise a turn at the tip of a loop section. The loss of one or two amino acids may simply result in shortening of the loop. All of these mutants contain three to six amino acid alterations within the randomized region as shown in Figure 18 with the respective levels of activity determined *in vitro*.

D. Effect of GCV and ACV on Mammalian Cells Expressing Mutant Thymidine Kinases

1. Subcloning into a Mammalian Expression Vector

Three mutant thymidine kinases are selected to evaluate for cell toxicity *in vivo* in the presence of ganciclovir or acyclovir. Mutant clones number 30, 75 and 132 and the wild-type thymidine kinase genes are restricted with *Nco*I and blunt-ended with Klenow. The gel isolated fragments (*Nco*I-blunt) are ligated to pCMV restricted with *Not*I and transformed into *E. coli* strain NM522. The wild-type TK gene in the wrong orientation relative to the CMV promoter is also used as a control. Qiagen column purified clones are sequenced to confirm orientation, sequence and the 5' junction region. The clones are designated pCMV, pCMV: TK-wrong, pCMV: TK, pCMV:30, pCMV:75 and pCMV:132.

2. Transfections

As an initial step to evaluate these mutants, the pCMV clones are introduced in the presence of a neomycin resistant marker plasmid (pSV2neo) into TS13 BHK tk$^-$ cells (baby hamster kidney cells) by calcium phosphate precipitation using a modified version of Chen and Okayama (*Molec. Cell. Biol. 7*:2745-2752, 1987).

Briefly, the cell transfections are performed as follows. Approximately 5 x 10^5 ts13 BHK tk$^-$ cells (ATCC CRL-1632) are plated on 100mm dishes in DMEM + 10% calf serum. For each transfection 1μg of pSV2neo and 10μg of a pCMV construct (pCMV, pCMV:TK-wrong (HSVTK in the wrong orientation relative to the promoter),

pCMV:HSVTK, pCMV:30, pCMV:75 or pCMV:132 DNA) in 0.25M $CaCl_2$ are mixed with 0.5ml 2 x BBS (see Chen and Okayama) and preincubated at 37°C at 2.5% CO_2 for 24 hours. The $CaCl_2$/DNA mix is added dropwise to the plates and mixed in well. After a 24 hour incubation at 37°C in a 2.5% CO_2 wet incubator, the cells are rinsed twice with Dulbecco PBS minus Ca/Mg and fed with fresh DMEM + 10% calf serum. Plates are incubated at 37°C with 6% CO_2. After 72 hours post-transfection the cells are split 1:3 and plated in DMEM + 10% calf serum containing G418 at 600μg/ml.

3. Selection and ED_{50} Determinations

The cells are selected on G418 (600μg/ml) at 37°C for 17 days. During this time the plates are pooled (for each DNA transfection) and split three times at a ratio of 1:3. Approximately 30-40 clones are selected in this manner for each transfected DNA containing a tk gene in the correct orientation. The pCMV and pCMV:TK-wrong transfections yielded between 130 and 140 clones each. G418 resistant clones are harvested, pooled and plated at a density of 2000 cells/well in 100μl DMEM + 10% calf serum and 200μl/ml G418 + 6% CO_2 in 96 well microtiter plates. A concentration range of either ganciclovir (0.125, 0.25, 0.5, 1, 2.5, 5, 7.5, 10 and 20μM) or acyclovir (0.5, 1, 2.5, 5, 10, 25, 50, 75 and 100μM) is added to each plate with 8 repeats of each concentration for each transfectant population (the no nucleoside analog controls each had 16 repeats). After three days in the presence of the nucleoside analog, Alamar Blue is added and 6 hours later the plates are scanned using a fluorometer as according to the manufacturer's protocol (Alamar Biosciences, Inc., Sacramento, CA). The plates are incubated a further 24 hours at 37°C and scanned again.

Determination of the fluorescence level of cells incubated in the presence of Alamar Blue directly relates to cell viability. Subtraction of the background fluorescence allows one to plot the cell survival versus the nucleoside analog concentration to determine to effective dose for killing 50% of the cells (ED_{50}). The survival curves are plotted with data from the second scan and are shown in Figures 19 (GCV) and 20 (ACV).

After 4 days on nucleoside analog the effective doses for 50% cell killing with GCV and ACV are determined from Figures 19 and 20 (see Table IV).

TABLE IV

ED_{50} GCV	fold over WT	ED_{50} ACV	fold over WT

WT	20μM	1	25μM	1
30	4.4μM	4.5	18μM	1.4
75	0.47μM	43	1.25μM	20
132	18μM	1.1	25μM	1

4. Enzyme Assays and Immunoblots

Cell extracts from 2.4 x 10^6 pooled transfectants are assayed for thymidine, ganciclovir and acyclovir activity. The levels of phosphorylation corresponded very well with the activities determined *in vitro* (rabbit reticulocyte lysate translation products) and the amount of protein expression as determined by western blot analyses. No immunoreactive band is seen in the lanes corresponding to pCMV or pCMV:TK-wrong (TK gene in the wrong orientation). Both the wild-type TK (pCMV:HSVTK) and pCMV:132 transfected cell lysates exhibited roughly equivalent band intensities. The immunoreactive band for pCMV:30 cell lysates is substantially more intense (5-10 fold) and that of pCMV:75 is approximately half the pCMV:HSVTK band intensity for the equivalent cell number.

5. Testing Mutants in Glioblastoma Cell Lines

Blunt-ended *Nco*I fragments isolated from pET23d:HSVTK, pET23d:30 and pET23d:75 are cloned into the *Hpa*I site of pLXSN (Miller and Rosman *BioTechniques* 7:980, 1989). Plasmid purification is done by Qiagen chromatography and the isolated DNA sequenced to confirm orientation and 5' junction regions. Stable transfectants of rat C6 glioblastomas (ATCC CCL-107) and a human glioblastoma cell line (SF767) are made as described above with the exception that pSV2-neo is not co-transfected since the neomycin phosphotransferase gene is encoded by pLXSN. Selection and analysis is essentially as described above.

E. Kinetic Analysis of Mutant Thymidine Kinases

1. Overexpression of Mutant and Wild-Type Enzymes

A single colony of pET23d:HSVTK, pET23d:30, pET23d:75 and pET23d:132 in BL21(DE3)tk cells is used to inoculate 5ml of M9ZB medium (1% tryptone, 0.5% NaCl, 1 x M9 salts, 1mM $MgSO_4$, 100μM $CaCl_2$ and 0.2% glucose) containing cabenicillin at 20μg/ml. The culture is incubated at 37°C overnight. The following day the 5ml culture is used to inoculate 1L M9ZB + cabenicillin at 20μg/ml and the culture allowed to grow at 37°C

to OD600 0.1. At that point IPTG is added to 0.4mM and the culture incubated a further 3 hours. The cells are chilled on ice, pelleted by centrifugation and the pellets washed once in cell wash buffer (50mM Tris, pH 7.5, 5mM EDTA, 10% sucrose) prior to freezing the pellets at -70°C. The next day the cells are resuspended in 12ml Buffer 1 (50mM Tris, pH 7.5, 10% sucrose, 2mM DTT, 5mM EDTA, 1mM PMSF) and the volume split into two 13ml Oakridge ultracentrifuge tubes. lml Buffer 1 containing 3mg lysozyme is added to each tube and the tubes left on ice for 1 hr. An additional lml Buffer 1 + protease inhibitor mix is added and the tube spun at 35krpm in a Sorvall T-1250 rotor at 4°C. The cleared supernatant is then aliquoted and frozen at -70°C.

2. Affinity Purification

A thymidylyl-sepharose column is used for a one step purification procedure (see Example 2). The lml bed volume column is prepared by passing 10ml Buffer 1 followed by 10ml Absorption Buffer (50mM Tris, pH 7.5, 10% sucrose, 2mM DTT, 25mM $MgAc_2$, 10mM ATP) over the column. Two ml of the cleared lysate is mixed with 2 ml of Absorption Buffer and passed through a 0.2μm filter. This mix is passed over the column 3 times. The column is washed with 5ml Absorption buffer three times and the 5ml fractions collected. To elute the enzyme, 3 - lml fractions of Thymidine Buffer (300mM Tris, pH 7.5, 10% sucrose, 2mM DTT, 50mM KCl, 600μM thymidine) is passed over the column and each lml fraction collected. The column is reactivated by loading on 10ml High Salt Buffer (50mM Tris, pH 7.5, 10% sucrose, 2mM DTT, 0.5M KCl) and 10ml 50mM Tris, pH 7.5. The column is stored in 50mM Tris pH 7.5 + 0.004% sodium azide. The extent of purification is monitored by Coomassie stained SDS:PAGE analysis and the concentration of purified protein determined using the BioRad Reagent (Bradford Reagent). The fraction containing TK protein is dialyzed against several liters of 50mM Tris, pH 7.5 10% sucrose, 2mM DTT at 4°C to remove thymidine.

3. Enzyme Kinetics

The kinetics of thymidine, ganciclovir and acyclovir phosphorylation by the wild-type, mutant 30 and 75 thymidine kinase enzymes with variant concentrations of radioactive nucleoside substrate are determined essentially as described in Example 3. K_m and V_{max} values are determined from double reciprocal plots and kcat values are calculated using the equation $V_{max} = k_{cat}[E_o]$ where $[E_o]$ is the total enzyme concentration. The BioRad reagent was used to determine the total enzyme concentration of purified thymidine kinase enzymes. Results are shown below in Table I.

TABLE V
Kinetic characterization of HSV-1 TK Mutants with thymidine, ACV and GCV as substrate

Substrate	thymidine			ganciclovir			acyclovir		
Enzyme	W.T.	75	30	W.T.	75	30	W.T.	75	30
K_m (μM)	.380	.950	13.3	47.6	10.0	333	417	23	455
k_{cat} (sec^{-1})	.230	.210	.003	.050	.050	.009	.008	.010	.001
k_{cat} (sec^{-1}) / K_m (μM)	.60	.22	2E-4	1E-3	4.8E-3	2.7E-5	1.8E-5	4.5E-4	2.1E-6

*Calculations of k_{cat} are per active site

EXAMPLE 9

PRODUCTION OF SECOND-GENERATION HSV-1 THYMIDINE KINASE MUTANTS HAVING AMINO ACID SUBSTITUTIONS IN RESIDUES 159-161 AND 168-169

This example describes the construction and analysis of a second generation of TK mutants, which are mutagenized at codons 159-161 and 168-169.

A. Isolation of Second Generation TK Mutants

As described above, mutants isolated from the LIF-ALL library show increased prodrug specificity compared to the wild-type TK (see also, Black et al., *Proc. Nat'l Acad. USA 93*:3525-3529, 1996). Using information from the ten most active mutants isolated from the LIF-ALL library, a new set of randomized oligonucleotides were synthesized and used to generate a second generation random library. Since the library was skewed to mutagenize codons encoding residues 159-161 and 169-170 to only represent a few amino acid substitutions, the library is considered to be semi-random.

Figure 21 shows the semi-randomized oligonucleotides used to generate the library and the possible amino acid substitutions expected. These complimentary and partially overlapping oligonucleotides (DMO2211 and 2212) were purified after separation on a denaturing gel. After annealing of the respective 3' ends, the oligonucleotides were extended with DNA polymerase to form a 100bp double-stranded DNA fragment. Following restriction with *Sac*I and *Kpn*I, the random fragments were ligated to pET23d:HSVTK-Dummy, which is described above and by Black et al., *Proc. Nat'l Acad. USA 93*:3525-3529, 1996). Vectors

containing the mix of random sequences were used to transform a thymidine kinase-deficient *E. coli*, and the transformed *E. coli* were plated on growth medium which requires the presence of a functional plasmid-borne TK. A total of 120 clones were picked and restreaked onto selective medium to confirm the phenotype. Individual colonies were used to inoculate selective medium aliquoted in 96-well plates (one clone/well). Cultures were examined for their sensitivity to different levels of GCV or ACV. Lysates of all 120 mutants were assayed for the ability to phosphorylate thymidine, ACV and GCV, using methods described above.

Seven mutants that demonstrated required activities were selected for further study. Table VI shows the deduced amino acid sequence of these seven mutants (SR11, SR26, SR39, SR4, SR15, SR32, SR53).

TABLE VI

Amino Acid Substitutions at Residues 159-161 and 168-169 in Second Generation Semi-Random Mutants

wild-type TK	L I F	D R H P I A A L L
SR11	- F L	F N
SR26	- F A	F -
SR39	I F L	F M
SR4	I L L	Y L
SR15	- F A	Y Y
SR32	- F V	V M
SR53	I F V	F Y

B. Analysis of Second Generation TK Mutants

1. In vitro analysis of Second generation semi-random mutants in cell lines

The seven mutants were subcloned into the mammalian expression vector, pREP8D7:dualGFP. This vector contains a constitutive metallothionin promoter, which drives the expression of green flourescent protein (GFP), and an RSV LTR promoter, which stimulated expression of the TK mutants. The vector also contains a histidinol resistance gene for selection of transformants. Purified vector DNA of these constructs was used to transfect BHK tk- cells by electroporation. The transfectants were selected by resistance to histidinol and sorted using FACS analysis for GFP expression. Pools of transfectants were then assayed

for sensitivity to GCV or ACV over a range of prodrug doses. In both ACV and GCV assays, six of the seven mutants revealed lower IC_{50} values than the wild-type TK transfectant pool. The remaining mutant transfectant pool (SR53) expressed low levels of TK protein which may account for its lower prodrug sensitivity. The results presented in Table VII show that mutants SR11, SR26, and SR39 are superior to wild-type TK or to mutant 75, using ACV as a substrate. Table VIII illustrates the IC_{50} values from Rat C6 kill curves with the SR11, SR26, and SR39 mutants.

2. In vivo analysis of second generation semi-random mutants in an in vivo mouse xenograft tumor model

Rat C6 glioblastoma cells were transfected with the stable expression vector pREP8D7:dualGFP as described above containing various TK mutants. Cells were transfected with either WT, SR39 or mutant 30 (LIF-ALL series) and sorted for comparable levels of GFP expression. Experiments were carried out to establish prodrug dosing levels for tumor ablation and efficacy of therapy. Nude mice (JAX Labs, Bar Harbor, Maine) were injected subcutaneously with 0.5 x10^6 transfected rat C6 cells. After 5 days, prodrugs (ACV and GCV) were administered twice a day for a further 5 days. Prodrug was given at either of two concentrations (shown as mg/kg). During this period and for an additional 6 days, tumor size was monitored by caliper measurement every other day. At the end of the period, mice were sacrificed and the tumors excised and weighed.

Data is presented in Figures 32, 33 (tumor diameter) and 34 (final tumor weight) and demonstrates that SR39 (as well as mutant 30) is a highly effective mutant and can cause significant tumor reduction using either ACV or GCV. The degree of in vivo tumor inhibition using both mutant 30 and SR39 are clearly superior to that of the wild-type enzyme. Further, data with SR39 and ACV suggest for the first time that ACV can function as an effective prodrug for suicide gene therapy.

TABLE VII

ICSO Values for ACV Kill Curves

Enzyme		ACV (μM)
TK		0.2
75		0.06
SR11		0.025
SR26		0.035
SR39		0.03

TABLE VIII

IC_{50} Values from Rat C6 Kill Curves

	$IC_{50}(\mu M)$			
	GCV	**relative to TK**	**ACV**	**relative to TK**
TK	5	1	>20	1
30	0.01	500	0.26	>77
75	>1	<5	>20	-
411	0.1	50	14	>1.4
SR11	0.15	33	6	>3
SR26	0.04	125	0.76	>26
SR39	0.107	294	0.11	>182

Enzyme kinetic analyses of purified SR11, SR26, and SR39 proteins were performed as described above. The results of these studies are summarized in Table IX.

TABLE IX

Kinetics of Semi-Random Library Mutants

	Km(μM)		
	Thymidine	**GCV**	**ACV**
TK	0.4	47	319
SR11	1.0	6.4	5.6
SR26	1.4	17.6	3.4
SR39	6.7	3.3	9.8

EXAMPLE 10

MUTAGENESIS OF A REGION WITHIN THE Q SUBSTRATE BINDING DOMAIN OF HSV-1 THYMIDINE KINASE

This example describes the construction and analysis of TK mutants that have been mutagenized in a region of the recently identified Q substrate binding domain.

A. Isolation of TK Mutants Having Modifications in the Q Substrate Binding Domain

To construct a dummy vector for insertion of the random sequences, a *Nar*I (or *Kas*I) site was introduced into pET23d:HSVTKII by site-directed mutagenesis, using primer DM01358 (5'-GTCTCGGAGGCGCCCAGCACC-3') within the wild-type thymidine kinase open reading frame at nucleotide position 276 from the ATG. The pET23d:HSVTKII vector is described by Black et al., *Proc. Nat'l Acad. Sci. USA 93*:3525-3529, 1996. Restriction of pET23d:HSVTK-Nar, which is the pET23d:HSVTKII vector with an engineered *Nar*I site, by *Sac*I and *Nar*I allowed removal of TK sequences and replacement by a 1kb *Nar*I/*Sac*I fragment from the vector, pLXSN. This vector was designated pET23d:HSVTK-Nar Dummy.

For the first random library, two oligonucleotides were synthesized containing the three non-wild-type nucleotides at a frequency of 9% (*i.e.*, the wild-type nucleotides were represented at 91% frequency) for the codons corresponding to residues 112-132. Figure 22 shows the sequences of oligonucleotides DMO-1860 and -1861, which are complementary and overlap. These oligonucleoties represent wild-type sequences. Random mutations were introduced by including non-wild-type nucleotides at a frequency of 9% for synthesis of regions presented in boldface type of DMO-1860 and -1861 oligonucleotides (*i.e.*, after the discontinuity indicated in each sequence). Figure 22 also outlines how the oligonucleotides were used in a PCR amplification to generate the correct-sized fragment. Briefly, an initial set of polymerase chain reactions (20 rounds) was performed to combine the four internal oligonucleotides (DMO-1860, DMO-1861, DMO-1893, and DMO-1894) into full-length product. A second PCR set (10 rounds) used the two smaller oligonucleotides, designated DMO-1895 and DMO-1896, to amplify the product and to add overhanging sequences for restriction cleavage. The product of this reaction was cleaved with *Kas*I and *Sac*I and ligated into the pET23d:HSVTK-Nar Dummy (*Kas*I/*Sac*I) vector. Following electroporation into BL21(DE3) tdk- *E. coli*, the cells were plated onto TK selection plates and scored for growth. All colonies were retested on fresh TK selection plates. Several hundred clones were sequenced and found to contain zero to six amino acid substitutions spanning the 20 amino acid region.

Two subsequent libraries were constructed using only one of the mutagenic oligonucleotides to increase the frequency of single amino acid changes. Several hundred TK positive clones were sequenced. Lysates from these mutants have been assayed for the ability to phosphorylate thymidine, acyclovir and ganciclovir, demonstrating that mutation within the Q substrate binding domain alters substrate specificity.

EXAMPLE 11

ISOLATION OF HUMAN AND MOUSE GUANYLATE KINASES AND CONSTRUCTION OF HSV-1 THYMIDINE KINASE AND GUANYLATE KINASE DUAL EXPRESSION VECTORS

This example describes the isolation of the human and mouse guanylate kinase genes and the vector construction for dual expression of herpes thymidine kinase and guanylate kinase.

A. Isolation of the Human Guanylate Kinase Gene

1. Isolation of the Human Guanylate Kinase Gene

Two oligonucleotides are designed to amplify the entire human guanylate kinase open reading frame. The following two oligonucleotides are synthesized by GenSet (La Jolla, CA): 5'-ACTACTGGAT[CCATGG]CGGGCCCCAGGCCTGTG-3', a 33-mer (SEQUENCE ID. NO. 26) and 5'-TACTACGGATCCTCAGGCGGCGGTCCTTTGAGC-3', a 33-mer (SEQUENCE ID. NO. 27). The *Bam*HI sites at each end are underlined and the *Nco*I site at the initiating methionine codon is shown in brackets. The bold nucleotide denotes a nucleotide alteration from the original sequence (GenBank accession number A11042). The human guanylate kinase gene is amplified from a cDNA library of human proliferating B lymphocytes stimulated with alpha-CD3. The resulting single band (~600bp) is restricted with *Bam*HI and cloned into pUC118 (*Bam*HI) to yield pUC118:Hugmk. The insert is sequenced in entirety (both strands) using the following set of oligonucleotides: 5'-CTGCTGAAGAGGCTGCTC-3' (18mer) (DMO 512) (SEQUENCE ID. NO. 28), 5'-ACACAGATGCGGTTTCATG-3' (19mer) (DMO 513) (SEQUENCE ID. NO. 29), 5'-CTGGACGTGGACCTGCAG-3' (18mer) (DMO 514) (SEQUENCE ID. NO. 30), 5'-GTTAATGATGACCACATC-3' (18mer) (DMO 515) (SEQUENCE ID. NO. 31), 5'-TGTAAAACGACGGCCAGT-3' (18mer) (M13 forward primer purchased from ABI) (SEQUENCE ID. NO. 32) and 5'-CAGGAAACAGCTATGACC-3' (18mer) (M13 reverse primer from ABI) (SEQUENCE ID. NO. 33). Sequence analysis revealed identity with the GenBank sequence except for the anticipated alteration at the *Nco*I site which results in a serine to alanine change (S2A) (Figure 24).

2. Northern Blot

8μg of total RNA from SP2/0 murine B lymphoma cells is prepared in 1 x MOPS buffer/75% formamide and heat denatured for 10 min at 55°C and loaded on a 1.2%

agarose gel in 1 x MOPS buffer. After transfer to nitrocellulose the blot is probed with the human gmk gene.

The 600bp *Bam*HI fragment is gel isolated from pUC118:Hugmk and is labeled using the random primer labeling kit from Amersham according to the manufacturer's instructions. The free radiolabel is removed by size exclusion chromatography. Following hybridization and washes the blot is exposed to X-ray film at -70°C for two days. Autoradiography of the northern blot reveals a single ~750nt RNA species. In a similar experiment using human poly A+ RNA from proliferating B lymphocytes, a single –750nt band is also observed.

B. Isolation of Mouse Guanylate Kinase Gene

1. Screening a Mouse cDNA Library

A lambda gt10 cDNA library of mouse 702/3 cells (B lymphomas) is probed using the human gene (same probe as used for northern blot analysis). The total number of plaques screened is 2 x 10^5 pfu. Nine independent lambda clones hybridized to the human probe and are plaque purified.

2. Subcloning and Sequence Analysis of Positive Clones

The *Eco*RI fragments from eight phage DNA preparations are gel isolated and subcloned into pUC118 restricted with *Eco*RI and dephosphorylated. The DNA insert sizes ranged from ~300bp to 1.2kb. Preliminary sequence analysis with primer (M13 forward primer) reveals that all clones began approximately 60bp 5' to the putative ATG start codon as determined by sequence alignment with the human and bovine guanylate kinase sequences and varied at their respective 3' ends. One representative clone (both strands) is completely sequenced using the following oligonucleotides: 5'-TGTGTCCCATACTACTACAAG-3' (21mer) (DMO 592) (SEQUENCE ID. NO. 34), 5'-TGAGAACTCAGCAGCATGCTC-3' (21mer) (DMO 594) (SEQUENCE ID. NO. 35), 5'-GTGCTAGATGTCGACCTA-3' (18mer) (DMO 595) (SEQUENCE ID. NO. 36), 5'-ACCTGGATAAAGCCTATG-3' (18mer) (DMO 674) (SEQUENCE ID. NO. 37), 5'-AAGCAGGCGCTCTCTCTGA-3' (19mer) (DMO 675) (SEQUENCE ID. NO. 38), 5'-CTATTTCTCATATGATGT-3' (18mer) (DMO 731) (SEQUENCE ID. NO. 39) and 5'-GTTACAGTGTCTCTAGAG-3' (18mer) (DMO 732) (SEQUENCE ID. NO. 40), 5'-TCCCCCACCTCCAGGC-3' (16mer) (DMO 748) (SEQUENCE ID. NO. 52), 5'-CTCAGTGTTGCCCAGTCG-3' (18mer) (DMO 749) SEQUENCE ID. NO. 53) and 5'-GCCGAAGATGCTGCTGTG-3' (18mer) (DMO 750)

SEQUENCE ID. NO. 54). The final murine guanylate kinase gene sequence is shown in Figure 25 with the deduced amino acids.

3. Introduction of a New Restriction Site

A novel *Nco*I restriction site is introduced at the start codon of the mouse guanylate kinase open reading frame as described in Black, M. E. and Hruby, D. E. (*J. Biol. Chem.* *265*:17584-17592, 1990). The mutagenic oligonucleotide used is: 5'-CTAGGTCCTG[CCATGG]CGTCCGCG-3' (24mer) (DMO 676) (SEQUENCE ID. NO. 41) with the *Nco*I site shown in brackets and the bold nucleotide denoting a C to G change. The resulting clone, pUC118:Mugmk-*Nco*I, is sequenced to confirm orientation and the 5' junction region.

C. Construction of Vectors for *in Vitro* Transcription and Translation Analysis

Both the human and murine guanylate kinase genes are subcloned into pET23d (see Example 8). The 600bp *Nco*I/*Bam*HI fragment from pUC118:Hugmk is gel isolated and directionally subcloned into pET23d (see Example 8) restricted with *Nco*I and *Bam*HI. The murine guanylate kinase gene is gel isolated as a ~800bp *Nco*I/*Eco*RI fragment using the introduced *Nco*I site at the ATG and the *Eco*RI site from the pUC118 3' polylinker region, and cloned into pET23d (see Example 8) restricted with *Nco*I and *Eco*RI. The resulting plasmids, pET23d:Hgmk and pET23d:Mgmk, are then used as templates for *in vitro* transcription and, the mRNAs produced, are used in a rabbit reticulocyte lysate cell free translation system as described in Examples 3 and 8. Enzyme assays to confirm full-length protein production and activity are as described in Agarwal et al. (*Methods in Enzymol.* *51*:483–490, 1978) with bovine guanylate kinase purchased from Sigma as a positive control.

D. Purification and Characterization of the Human and Mouse Civanylate Kinases

1. Expression Vector Construction

The pET23d vector (Novagen, Madison, WI) is used as the vector backbone for the construction of pET:HT. This vector contains a 6 histidine residue peptide followed by a thrombin cleavage site to allow for the expression of a removable histidine tag fused to the N terminus of the target gene product. Synthesis of the 6 his-thrombin fusion encoding region is done by PCR amplification of the promoter region of pET23d and extension using the following primers in three sequential PCR amplification steps. 5'-ACTACTACTA GATCTCGATC CCGCGAA-3' (27mer) (DMO 604) (SEQUENCE ID. NO. 42)

5'-ATGATGATGA TGATGGCTGC TAGCCATAGT ATATCTCCTT C-3' (41mer) (DMO 605) (SEQUENCE ID. NO. 43) 5'-CGGCACCAGG CCGCTGCTGT GATGATGATG ATGATGGCT-3' (39mer) (DMO 606) (SEQUENCE ID. NO. 44), 5- AGTAGTAT[CC ATGG]AGCTGC CGCGCGGCAC CAGGCCGCTG CT-3' (42mer) (DMO 607) (SEQUENCE ID. NO. 45). Sequence DMO 604 is annealed to the *Bgl*II region of pET23d in all PCR amplification steps. Sequence DMO 605 is annealed to the region corresponding to the *Nco*I site in a 3' to 5' orientation and results in the loss of the *Nco*I site due to a nucleotide mutation shown in bold in the sequence above. Subsequent amplifications with sequence DMO 606 or DMO 607 in the 3' to 5' orientation are paired with sequence DMO 604 to extend the sequence for the addition of 6 histidine codons and a thrombin cleavage site. A new *Nco*I site is also introduced with sequence DMO 607 as shown in brackets above. The final *Bgl*II/*Nco*I fragment is cloned into pET23d at the corresponding sites to create pET:HT. pET:HT is sequenced to confirm correct synthesis and insertion. The amino acid sequence of the new vector fusion peptide is: M A S S H H H H H H S S G L V P R G S S M (*Nco*I site) (SEQUENCE ID. NO. 46) with the thrombin cleavage recognition site underlined. Cleavage with thrombin is between the arginine and glycine residues.

2. Overexpression in *E. coli* and Affinity Purification

Methods for overexpression and analysis are as in Example 8. Affinity purification using His-Bind Resin (Novagen, Madison WI) is performed according to the manufacturer's instruction. Thrombin is used to cleave off the terminal 17 amino acids to leave three amino acids N-terminal to the guanylate kinase initiating methionine. The leader peptide is then removed by passing the cleavage mix over the His-Bind column a second time.

3. Enzyme Kinetics

The K_m, V_{max} and K_{cat} values for guanylate, GCV-monophosphate and acyclovir-monophosphate are determined using purified human and mouse guanylate kinases. In addition to using the assay protocol described in Agarwal et al. (*Methods in Enzymol. 51*:483-490, 1978), the nucleotide products generated from assays performed with radionucleotide substrates are analyzed by thin layer chromatography and scintillation counting.

E. Expression of Human and Murine Guanylate Kinases in Mammalian Cells

1. Vector construction

Both human and murine guanylate kinase genes are cloned into a modified pREP8 vector. Briefly, for construction of the modified pREP8 (pREP8-7kb), pREP8 (Invitrogen) is digested with *Bst*EII and *Xba*I, filled in with Klenow and religated. The resulting plasmid, pREP8-7kb, no longer encodes EBNA-1 or the EBV origin of replication (oriP). Both guanylate kinases, pET23d:hgmk and pET23d:mgmk (described above) are restricted with *Nco*I, blunt-ended and then digested with *Bam*HI to yield a -600bp *Nco*I (blunt)-*Bam*HI fragment after gel purification. These are ligated to pREP8-7kb that has been digested with *Hin*DIII (blunt-ended) and *Bam*HI. The new plasmids are designated pREP8-7:hgmk and pREP8-7:mgmk.

2. Isolation of stable transfectants expressing HSVTK

BHKtk-(ts13) cells are transfected with pCMV, pCMV:TK, pCMV:30 and pCMV:75 DNA in the presence of pSV2-neo (10:1 ratio) as described in Example 8. Approximately 10-20 individual clones from each pCMV DNA transfection are isolated under 1mg/ml G418 selection. As in example 8, about 2 x 10^6 cells per clone are examined for TK expression level by western blot using polyclonal anti-TK serum.

Expression of TK clone C3 is very high, whereas 75 D4 and 30 A2 are less than half the TK expression level of C3. 75 D2, D3 and D4 protein expression ranged from very low, low to moderate, respectively.

3. Sensitivity of clones to GCV or ACV

Clones are assayed for sensitivity to GCV and ACV as described in Example 8. Sensitivity to GCV and ACV is dependent on the level of protein expression. This can clearly be seen with the 75 clones, D2, D3 and D4 where the highest expression clone D4 is the most sensitive, D3 is less so and D2 is even less sensitive than D3 to prodrugs. (Figures 26, 27)

4. Transfection of TK-expressing cells with pREP8-7 guanylate kinase constructs

pREP8-7, pREP8-7:hgmk and pREP8-7:mgmk are used to transfect BHK tk, TK-transfected clone C3 and 75-transfected clone D4. Histidinol is used to select pools of stable transfectants and to isolate individual clones.

Protein expression levels of guanylate kinase in the different pools is determined by immunoblot analysis. Briefly, 5 μl of 2 x 10^6 cell pellet lysates (200 μl) are

subjected to electrophoresis and transferred to nitrocellulose. Polyclonal anti-guanylate kinase serum (at a 1:5,000 dilution) and TK antiserum (at a 1:10,000 dilution) is utilized to detect the resultant protein bands.

5. Sensitivity of guanylate kinase transfectant pools to GCV and ACV in TK expressing clones

As in Example 8, pools of transfectants are placed in 96 well microtiter dishes at 1000 cells/well. Eight replicates are incubated for three days in the presence of various GCV or ACV concentrations.

As can be seen in Figures 28 and 29, the level of prodrug sensitivity is related to the level of TK protein expression and the presence of guanylate kinase. Guanylate kinase expression in the presence of wild-type TK demonstrates approximately 2 fold increased sensitivity to ACV relative to TK expression alone. Despite half the expression level of wild-type TK, sensitivity to ACV by gmk + 75 D4 expressing cells is 6-7 times greater than that of TK expressing cells.

F. Construction and Analysis of Dual Expression Vectors *in Vivo*

The HSV1 tk gene is cloned into the *Hpa*I site of pLXSN (Miller and Rosman, *BioTechniques* 7:980-990, 1989) as a *Nco*I (blunt-ended) fragment and the orientation determined by restriction mapping. This places the HSV-1 tk gene behind the MoMLV LTR promoter. The neomycin phosphotransferase gene is replaced by the guanylate kinase gene (human or mouse) as a *Bam*HI (blunt-ended) fragment such that guanylate kinase gene expression is driven off the SV40 promoter. In addition, vectors are constructed where the tk and gmk gene order is reversed such that the tk gene is expressed from the SV promoter and gmk is expressed from the LTR promoter. Vector constructs with individual genes (tk or gmk) are also constructed. Furthermore, expression vectors containing HSV-1 tk mutants in place of the wild-type HSV-1 tk genes are also constructed.

As in Example 8, plasmid DNA from the constructs described above are used to transfect ts13 BHK tk- cells, SF767 human glioblastoma cells, and rat C6 glioblastoma cells in the presence of a marker plasmid (pSV2-neo) to enable the selection of transfectants on G418.

Selection of stable transfectants and assays for increased sensitivity to ACV and GCV are as described in Example 8.

EXAMPLE 12

CONSTRUCTION AND ANALYSIS OF GUANYLATE KINASE - THYMIDINE KINASE FUSION PROTEINS

This example illustrates the production and analysis of several fusion proteins that have both guanylate kinase and thymidine kinase activities.

A. Construction of Fusion Proteins

Use of a fusion protein for gene therapy would not only negate the requirement for two promoters and the associated reduction in prodrug activation due to the differences in promoter strength, it would also allow expression of two enzyme functions from a single promoter and a single cistron. Accordingly, fusion proteins are advantageous for gene therapy vectors which cannot tolerate large pieces of foreign DNA, such as AAV vectors.

Two fusion proteins have been constructed that contain both wild-type HSV-1 TK and murine guanylate kinase (gmk) sequences. These proteins differ in the number of residues at the fusion site. Both fusion constructs can be over-expressed in *E. coli* from pET23d backbone vectors. In both vectors, guanylate kinase was located adjacent to the promoter with TK fused to the *Msc*I site at the 3' end of gmk which removes the two C-terminal amino acids. One fusion was constructed such that the first nine amino acids of TK are absent (pET23d:gmlc/TK-trunc). The other fusion contains the entire TK amino acid sequence (pET23d:gmk/TK-fl). Maps of these constructs are illustrated in Figure 30.

Six additional fusion proteins have been constructed in which the wild-type TK sequence of pET23d:gmk/TK-fl is replaced by TK mutant 30, mutant 75, mutant 411, SR11, SR26 or SR39 sequences. These fusion proteins were over-expressed in BL21(DE3) tk- cells.

B. Analysis of Fusion Proteins

All of the above constructs were cloned into pREP8D7:dualGFP, as described above. These vectors were used to transfect BHK tk- cells and transfectants were selected on the basis of resistance to histidinol. Further screening for GFP expression was performed by FACS analysis. In addition, the gmk/TK-fl construct was used to transfect rat C6 glioma cells and positive clones/pools were selected as described above. A ganciclovir dose response curve comparing gmk/TK-trunc to wild-type TK in rat C6 cells is shown in Figure 31. This curve demonstrates a 100-fold difference in IC_{50} between the two enzymes with the fusion protein being the superior one.

Both wild-type TK-gmk fusion proteins were over-expressed in *E. coli* and purified to homogeneity using affinity chromatography. Michaelis-Menten kinetics for both thymidine kinase and guanylate kinase activities were examined with both fusion proteins, and the results are shown in Table X. The thymidine kinase activity is similar to wild-type levels. However, gmk function is impaired 3.8 to 5.8 fold in the fusion protein constructs compared to wild-type gmk. Nevertheless, the fusion proteins exhibited both guanylate kinase and TK activities.

Table X

Kinetic Analysis of Fusion Proteins

	Km (μM)			
	gmk	**gmk/TK-trunk**	**gmk/TK-fl**	**TK**
GMP	25	95	146	-
dGMP		218	359	-
thymidine	-	0.67	0.5	0.3

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.